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TI Alkaloids from the fungus Penicillium aurantio-virens Biourge and some aspects of their formation
AU Solov'eva, T. F.; Kuvichkina, T. N.; Baskunov, B. P.; Kozlovskii, A. G.
CS Inst. of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushkino, 142292, Russia
SO Mikrobiologiya (1995), 64(5), 645-650

TI Alkaloids of Stipa robusta (sleepygrass) infected with an Acremonium endophyte
AU Petroski, Richard J.; Powell, Richard G.; Clay, Keith
CS Natl. Cent. Agric. Util. Res., Agric. Res. Serv., Peoria, IL, 61604, USA
SO Nat. Toxins (1992), 1(2), 84-8

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TI Alkaloid composition of Penicillium palitans and Penicillium oxalicum
AU Vinokurova, N. G.; Reshetilova, T. A.; Adanin, V. M.; Kozlovskii, A. G.
CS Inst. Biochem. Physiol. Microorg., Pushchino, USSR
SO Prikl. Biokhim. Mikrobiol. (1991), 27(6), 850-5

TI Enzymic transformation of chanoclavine-I by Penicillium sizovae F-209 cell-free extract
AU Markelova, N. Yu.; Kozlovskii, A. G.
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ERGOT TOXICITY FROM ENDOPHYTE-INFECTED GRASSES A REVIEW.
AU BACON C W; LYONS P C; PORTER J K; ROBBINS J D
CS TOXICOL. BIOL. CONSTIT. RES. UNIT, R.B. RUSSELL AGRIC. RES. CENT., USDA-ARS, ATHENS, GA.
SO AGRON J, (1986) 78 (1), 106-116.

Ergot alkaloids. Isolation of N-demethylchanoclavine-II from Claviceps strain SD 58 and the role of demethylchanoclavines in ergoline biosynthesis
AU Cassady, John M.; Abou-Chaar, Charles I.; Floss, Heinz G.
CS Dep. Med. Chem. Pharmacogn., Purdue Univ., Lafayette, Indiana, USA
SO Lloydia (1973), 36(4), 390-6

Enzymic conversion of chanoclavine I
AU Erge, D.; Maier, W.; Groeger, D.
CS Inst. Biochem. Pflanzen, Halle/Saale, E. Ger.
SO Biochem. Physiol. Pflanz. (1973), 164(3), 234-47

TI Production of alkaloids and related substances by fungi. III. Isolation of chanoclavine I and two new interconvertible alkaloids, regulovasine A and B, from Penicillium cultures
AU Abe, Matazo; Ohmomo, Sadahiro; Ohashi, Tsutomu; Tabuchi, Takeshi
CS Tokyo Univ. Educ., Tokyo, Japan
SO Nippon Nogei Kagaku Kaishi (1969), 43(8), 575-82
CODEN: NNKKAA
DT Journal

Ergot alkaloids from plants

Ergot Alkaloids. Isolation of *N*-Demethylchanoclavine-II from *Claviceps* Strain SD 58 and the Role of Demethylchanoclavines in Ergoline Biosynthesis

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ABSTRACT.—A new trace alkaloid was isolated from *Claviceps* strain SD 58 and identified as the so far unknown (−)-*N*-demethylchanoclavine-II. Neither this compound nor *N*-demethylchanoclavine-I-17-T prepared by chemical demethylation of chanoclavine-I-17-T was incorporated significantly into elymoclavine by the ergot fungus. This suggests that these demethylchanoclavines are not precursors of the chanoclavines, but more likely arise from the latter by biological demethylation.

In the course of various experiments with *Claviceps* strain SD 58 we have noted the presence of an unidentified ergoline-like compound, which we have designated substance *Z* (1, 2). In this paper we wish to report the purification of substance *Z* and its identification as the hitherto unknown *N*-demethylchanoclavine-II.

The biogenesis of ergot alkaloids (*cf.* 3, 4 for reviews) involves the transfer of an intact methyl group from methionine to the nitrogen atom derived from the amino group of tryptophan (5). The exact stage at which this methylation takes place has not been determined, but the fact that *N*-methyltryptophan and *N*-methyltryptamine are not intermediates in ergoline biosynthesis (6) suggests that it must occur between 4-dimethylallyltryptophan and chanoclavine-I, the first known methylated alkaloid in the pathway. We had tacitly assumed that the methylation takes place at the chanoclavine stage, since this would allow for participation of pyridoxal phosphate in the decarboxylation and formation of ring C (1), which would be mechanistically plausible and is supported by some circumstantial evidence (7). The data reported in this paper, however, indicate that *N*-demethylchanoclavine-I is not converted into tetracyclic ergolines by *Claviceps* strain SD 58.

EXPERIMENTAL[2]

CHROMATOGRAPHY (*cf.* 1).—The following solvent systems were used with 5 x 20 x 0.025 cm Brinkmann Silplate 52 pre-coated silica gel plates or 20 x 20 x 0.05 cm activated silica gel G plates: AED, acetone–ethyl acetate–*N*,*N*-dimethylformamide (5:5:1); CMA, chloroform–methanol (9:1) in ammonia atmosphere; CTBA, chloroform–*tert*-butanol (3:1) in ammonia atmosphere. Ehrlich's reagent (*cf.* 1) was used to visualize alkaloids on thin-layer plates. For recovery of alkaloids from tlc plates, the bands were scraped off and eluted with acetone–2% succinic acid solution (2:1). The acetone was removed in a stream of nitrogen, the aqueous phase made alkaline with ammonia and the alkaloid extracted into methylene chloride. High pressure liquid chromatography was done on a DuPont 830 liquid chromatograph at 350 psi using an AAX column and borate buffer, pH 9.6, at a flow rate of 0.6 ml/min.

ISOLATION OF COMPOUND *Z*.—*Claviceps species*, strain SD 58 was grown in 100 ml shake cultures to an age of 15–20 days as described previously (8, 1). Culture filtrates assaying 50–80 mg alkaloid per 100 ml were pooled in batches of 15 cultures, made alkaline to pH 11 with ammonia and extracted 7 times with chloroform or chloroform–isopropanol (3:1). The extracts from four such batches were combined and evaporated to dryness in a vacuum. The residue was dissolved in 2% aqueous succinic acid, the solution washed 3 times with methylene chloride,

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[2]Uv spectra were recorded on a Perkin-Elmer Coleman 124 spectrophotometer, ord spectra on a Cary 60 ord/cd spectrometer, and mass spectra were obtained at 70eV and inlet temperatures of 130–200° on a Hitachi RMU-6 low resolution or a CEC 21–110 high resolution mass spectrometer. Radioactivity determinations were done by liquid scintillation counting in a Beckman LS-100 instrument using methanol as solvent and PPO + dimethyl POPOP in toluene as scintillator solution. Counting efficiencies were determined by the addition of internal standard. Radioactivity on chromatograms was located by scanning in a Packard Model 7401 radiochromatogram scanner. The alkaloids were quantitated by colorimetry using van Urk's reagent (*cf.* 1).

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made alkaline with ammonia to pH 11 and extracted seven times with methylene chloride. This extract, which contained the alkaloids, was dried over anhydrous sodium sulfate, concentrated to 120 ml in a vacuum and left in the refrigerator overnight. The solution was then filtered through a fine sintered glass funnel to remove the crystallized elymoclavine, which was washed with 3 ml cold methylene chloride. The filtrate and washings were then passed through a column (2 x 75 cm) containing 100 g of Al_2O_3 (Brockmann activity II–III) suspended in methylene chloride. The column was first eluted with methylene chloride containing 2% methanol until no more isochanoclavine-I could be detected in the eluate (1100–1200 ml or more). These fractions contain agroclavine, elymoclavine and isochanoclavine-I, as well as a large portion of the chanoclavine-II and a smaller portion of chanoclavine-I. The elution was continued with methylene chloride containing 10% methanol to give two more fractions. The first 1000 ml contained the bulk of chanoclavine-I, some chanoclavine-II and substance Z and the following 1500–1800 ml had mainly substance Z and another compound of slightly lower R_F in the AED system. These two fractions were evaporated and streaked, respectively, on twenty-five and five 20 x 20 x 0.05 cm silica gel G plates. The plates were developed twice in the AED system, allowing the solvent front to travel at least 15 cm. The band containing substance Z was scraped off and the alkaloid was eluted from the gel. This material was then rechromatographed in the CMA system. At this point the alkaloid was chromatographically homogeneous. It had the following characteristics: MW 242.1430 (calc. for $C_{15}H_{18}N_2O$ (242.1419); uv, λ max (MeOH) 223, 274, 283, and 294 nm; ord, ϕ310nm −10,273 (MeOH); color with Ehrlich's reagent, grey, turning blue.

DEMETHYLATION OF CHANOCLAVINE-I.—Sixty-four mg of chanoclavine-I was dissolved in 15 ml of anhydrous acetone under nitrogen, 200 mg of diethylazodicarboxylate in 10 ml absolute ether was added and the mixture was refluxed under nitrogen for 12 hours. The reaction mixture was partitioned between ether and 2% aqueous succinic acid, the aqueous phase was made alkaline with ammonia and the alkaloid extracted into ether. The crude material (40 mg, consisting mainly of *N*-demethylchanoclavine-I and unreacted starting material) was chromatographed on a small column of 5 g of Al_2O_3 Brockmann, which was successively eluted with chloroform, chloroform with 2% methanol and chloroform containing 10% methanol. Demethylchanoclavine-I, together with small amounts of chanoclavine-I, was eluted with the latter solvent. It was further purified by chromatographing it twice on preparative tlc plates in the CMA system to give 3 mg of chromatographically homogeneous material, which showed the following characteristics: MW 242.1413 (calc. for $C_{15}H_{18}N_2O$ 242.1419); uv, λ max (MeOH) 223, 273, 283, and 293 nm; ord, ϕ310nm −3,255 (MeOH); color with Ehrlich's reagent, grey, turning blue.

DEMETHYLATION OF DIHYDROELYMOCLAVINE.—Five mg of dihydroelymoclavine (9) in 1.5 ml acetone and 15 mg diethylazodicarboxylate in 1 ml ether were mixed, left at room temperature under nitrogen overnight and then refluxed for one hour. After the usual workup, the reaction product was compared chromatographically with substance Z in the CMA system. It contained in addition to unreacted starting material (R_F 0.39), a new compound (R_F 0.14) which clearly differed from substance Z (R_F 0.34).

METHYLATION OF *N*-DEMETHYLCHANOCLAVINE-I AND SUBSTANCE Z.—One-half mg of *N*-demethylchanoclavine-I or substance Z was dissolved in 1 ml methanol and stirred with 5 drops of methyl iodide on a hot plate stirrer at 40° for 30 to 60 min. The mixture was then concentrated and aliquots were spotted on 5 x 20 x 0.025 cm precoated silica gel plates along with reference alkaloids chanoclavine-I, chanoclavine-II and isochanoclavine-I. The plates were developed in the CMA or CTBA systems. The reaction mixtures contained in varying proportions unreacted starting material, the monomethylation product and the dimethylation product. The conversion products from *N*-demethylchanoclavine-I co-chromatographed with chanoclavine-I and *N*-methylchanoclavine-I, the conversion products from substance Z differed from these two reference compounds and from isochanoclavine-I, but the major one of them clearly co-chromatographed with chanoclavine-II.

PREPARATION OF *N*-DEMETHYLCHANOCLAVINE-I-17-T.—Fifty-five mg of chanoclavine-I-aldehyde (10) was dissolved in 4 ml methanol which had been freshly distilled over $NaBH_4$. The solution was cooled in an ice bath and tritiated $NaBH_4$ (25 mCi, 502 mCi/mmole) was added as a solid, using some of the solution to rinse the ampule. After 90 min. at 10°, 10 mg of cold $NaBH_4$ were added and the solution was left for another hour. The reaction mixture was worked up by removing the methanol in a vacuum, partitioning the residue between ether and water, washing the ether layer twice with water, followed by drying with Na_2SO_4 and evaporation of the ether. The residue was chromatographed on a column of 6g of Al_2O_3 (Brockmann) which was eluted first with $CHCl_3$ and then with $CHCl_3$ containing 2% methanol. The latter solvent eluted chromatographically pure tritiated chanoclavine-I (45 mg, 4mCi). Eleven mg of this material was dissolved in 10 ml dry acetone and refluxed with 40 mg diethylazodicarboxylate in 10 ml abs. ether for 10 hours under nitrogen. When tlc of the reaction mixture showed less than 5% demethylation product to be present, another 40 mg of diethylazodicarboxylate was added and refluxing under nitrogen was continued for another 4 hours. At this time considerable decomposition was evident by tlc and the reaction mixture was worked

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up by evaporating the solvent and partitioning the residue between ether and 2% succinic acid solution. The aqueous phase was made alkaline with NH_4OH and extracted with 7 x 15 ml ether. The extract was dried over Na_2SO_4, concentrated and chromatographed on seven 5 x 20 x 0.025 cm precoated silica gel plates in the CMA system. Elution of the *N*-demethylchanoclavine-I band gave 1.5 mg (18 μCi) of material which according to analysis by radiochromatography still contained about 1% tritiated chanoclavine-I. A portion of this material (6.8 μCi) was rechromatographed in the same way to give 2.5 μCi of pure *N*-demethylchanoclavine-I-17-T, which contained less than 0.1% of radioactive contaminants.

FEEDING EXPERIMENT.—*N*-Demethylchanoclavine-I-17-T and chanoclavine-I-17-T (spec. act. 23 mCi/mmole, 1.67 μCi and 1.55 μCi, respectively) were each dissolved in 1 ml 2% aqueous succinic acid, filtered through a millipore filter and added each to one 5-day-old 100 ml shake culture of *Claviceps* strain SD 58. After incubation for another 5 days the cultures were harvested, the alkaloid titer was determined and elymoclavine was isolated by column chromatography and purified by recrystallization from methanol as described previously (8). In the *N*-demethylchanoclavine-I experiment, the specific activity of elymoclavine after the 2nd, 3rd and 4th crystallization was 111, 81 and 68 dpm/mg, in the chanoclavine-I experiment the figures after the 3rd, 4th and 5th crystallizations were 3630, 3120 and 3200 dpm/mg.

UPTAKE EXPERIMENT.—*N*-Demethylchanoclavine-I-17-T (1.35 μCi) was fed to one 5-day-old shake culture of strain SD 58. The flask was returned to the shaker and part of the culture (41 ml) was harvested 2 hours later, the rest (47 ml) after 5 hours. The mycelia were filtered with suction, washed throughly with water, weighed, freeze-dried, weighed again and each soaked overnight with 10 ml of methanol. After centrifugation, an aliquot of the methanol solution was counted for radioactivity. It was found that after 2 and 5 hours, 3.6% and 3.1% of the total added radioactivity resided in the mycelium. From the difference of mycelial fresh and dry weight the amount of intracellular fluid can be estimated (3.4 ml) and from this figure and the total culture volume (89 ml) it follows that upon complete equilibration of the labeled material between intracellular and extracellular fluid about 3.9% of the total radioactivity should reside in the mycelium.

RESULTS

Substance Z was obtained in a variety of radioactive feeding experiments with *Claviceps* spec., strain SD 58, as an Ehrlich-positive material of usually lower R_F value than the chanoclavines (1, 2). Analyses of the samples obtained in these biosynthetic experiments indicated that the compound incorporated radioactivity from tryptophan-G-^{3}H and mevalonate-2-^{14}C and that the *pro*-4*R* and *pro*-5*S* hydrogens, but not the *pro*-5*R* hydrogen, of mevalonate were retained in its formation. This pattern of biosynthetic labeling conforms to that of the common tricyclic and tetracyclic clavine alkaloids (1, 2, 11). For the elucidation of its structure, substance Z was isolated preparatively from cultures of *Claviceps* strain SD 58 by removal of the bulk of the elymoclavine from the alkaloid extract, column chromatography on alumina followed by preparative *tlc* on silica gel as described in the experimental section. This procedure gives about 1–2 mg of chromatographically pure, non-crystalline material from sixty 100-ml cultures.

The uv spectrum (λmax (MeOH) 223, 274, 283 and 294 nm) shows the presence of an unconjugated indole chromophore. The molecular formula $C_{15}H_{18}N_2O$ followed from the high resolution mass spectrum, which indicated a molecular weight of 242.1430 (calc. 242.1419). The mass spectrum (Figure 1a) showed strong peaks at *m/e* 154, 167, 169 and 182, typical of tricyclic and tetracyclic ergolines. The absence of an M–1 peak and the presence of strong peaks at M–18 and M–19 (*m/e* = 224, 223) closely parallels the fragmentation pattern observed for chanoclavine-I (Figure 1b). Thus it was considered that substance Z might be a homolog of the chanoclavines lacking the *N*-methyl group. As another possibility, an *N*-demethyl-dihydroelymoclavine would also fit the observed molecular formula. However, the mass spectral fragmentation

EXPLANATION OF FIGURE 1

FIG. 1. *Mass spectra of* (a) *substance Z* (N-*demethylchanoclavine-II*), (*b*) *chanoclavine-I* (c) N-*demethylchanoclavine-I and* (d) *dihydroelymoclavine.*

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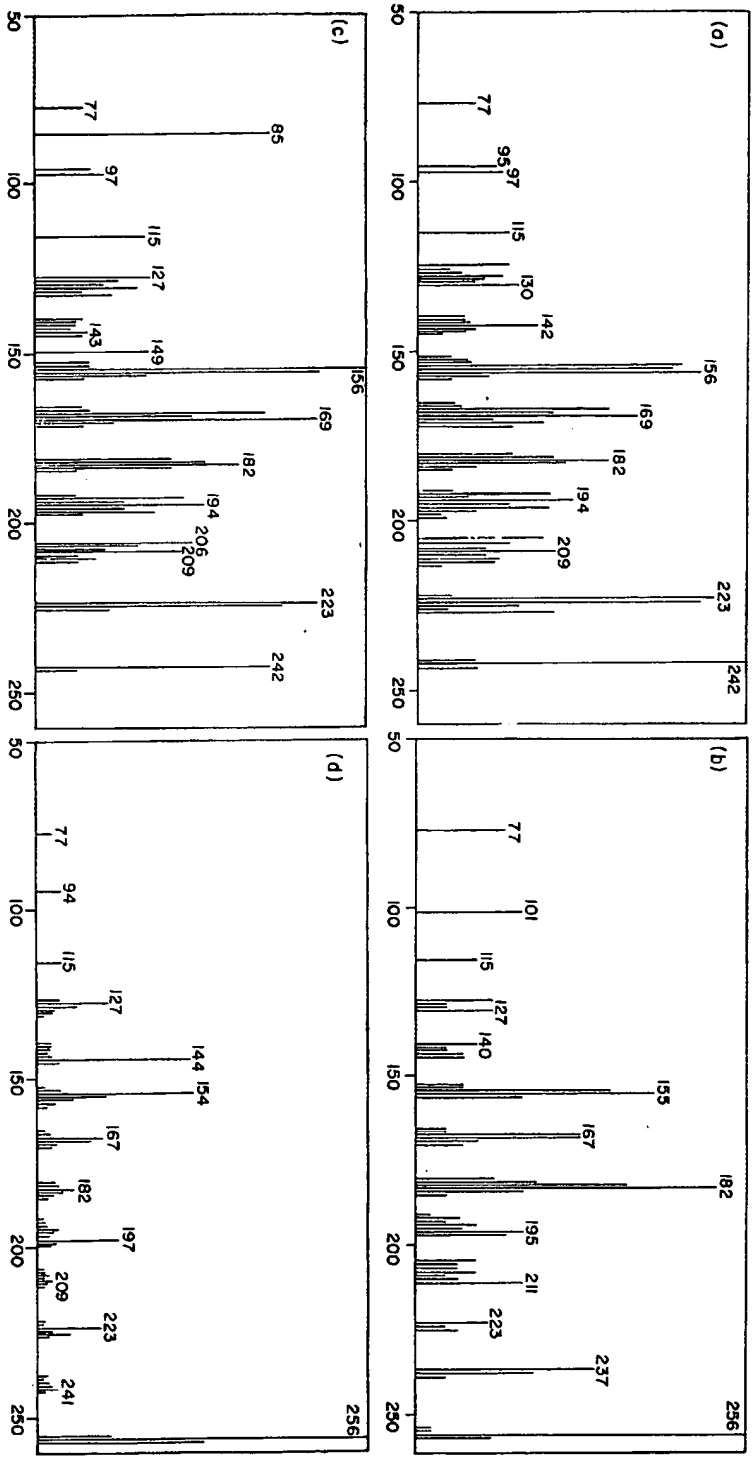

oclavine-II), (*b*) *chano-*
oelymoclavine.

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pattern of dihydroelymoclavine differs considerably from that of substance Z (Figure 1d).

In view of the small amount of substance Z available we attempted to establish a correlation by demethylation of known alkaloids. Fehr (12) has shown that diethylazodicarboxylate can be used to effect the removal of methyl groups from tertiary and secondary amines in the clavine series. Use of this method with dihydroelymoclavine and chanoclavine-I under slightly more vigorous conditions gave in each case a new product of lower R_F value. The material from dihydroelymoclavine clearly differed in chromatographic mobility from substance Z, but the demethylation product from chanoclavine-I co-chromatographed with substance Z on tlc in the two solvent systems used and in high-pressure liquid chromatography. This reaction product was purified and the assumption that it was *N*-demethylchanoclavine-I was confirmed by its uv and mass spectra and by remethylation to chanoclavine-I. Whereas the chromatographic mobilities in many systems and the overall mass spectral fragmentation patterns of *N*-demethylchanoclavine-I and substance Z were essentially identical, a detailed comparison of the mass spectra of the two compounds run under

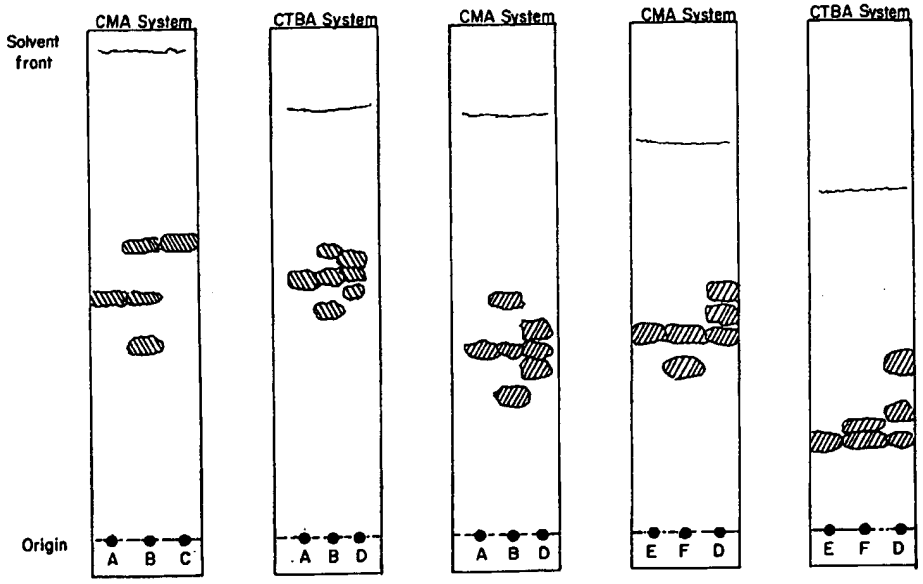

FIG. 2. *Chromatographic comparison of the methylation products from substance Z and N-demethylchanoclavine-I with the chanoclavine isomers.* A: *chanoclavine-I;* B: *methylation product from N-demethylchanoclavine-I;* C: *N-methylchanoclavine-I;* D: *chanoclavine-II, chanoclavine-I and isochanoclavine-I (in order of increasing R_F);* E: *chanoclavine-II;* F: *methylation product from substance Z.*

identical conditions (Figure 1a and c) revealed significant differences in the relative intensities of the various peaks. This indicated that the two compounds were very closely related but not identical, suggesting the possibility that they might be stereoisomers. This notion was further supported by the ord spectra, which showed negative plain curves. The molecular rotation of substance Z (ϕ310nm = −10,273, MeOH) relates to that of *N*-demethylchanoclavine-I (ϕ310nm = −3,255, MeOH) in about the same way as that of (−)-chanoclavine-II (ϕ312nm = −15,100, pyridine) (1) to that of chanoclavine-I (ϕ310nm = −3,960 pyridine), and thus we concluded that substance Z was probably (−)-*N*-demethylchanoclavine-II. This conclusion was proven to be correct by methyla-

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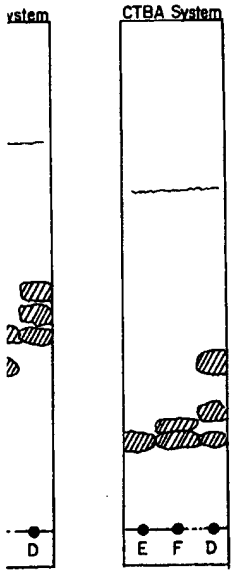

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tion of substance Z and chromatographic comparison with all the known chanoclavine isomers (Figure 2).

Further experiments dealt with the question of whether the *N*-demethylchanoclavines might be intermediates in the formation of tetracyclic ergolines. An early experiment with biosynthetically tritiated substance Z had shown that it was not efficiently incorporated into elymoclavine, which is not surprising in view of its stereochemistry and in view of the fact that (−)-chanoclavine-II is not a precursor of tetracyclic ergolines (2). *N*-Demethylchanoclavine-I on the other hand could well be a precursor of chanoclavine-I and hence of the tetracyclic ergolines. To test this point we prepared a sample of *N*-demethylchanoclavine-I-17-T by demethylation of chanoclavine-I-17-T available from earlier experiments (10). This material was purified chromatographically to radiochemical homogeneity and particular care was taken to ascertain the virtual absence (<0.1%) of unreacted chanoclavine-I-17-T. A sample was then fed to a 5-day-old shake culture of *Claviceps* strain SD 58 and a sample of chanoclavine-I-17-T was fed to a parallel culture. Five days later these cultures were harvested, elymoclavine was isolated from the extracted total alkaloids by column chromatography on alumina in the usual way (8) and recrystallized from methanol to constant specific radioactivity or until the material had been used up. As shown in table 1, in the experiment with chanoclavine-I-17-T the elymoclavine

TABLE 1. *Incorporation of chanoclavine-I and N-demethylchanoclavine-I into elymoclavine by* Claviceps *species, strain SD 58.*

	Precursor fed	
	Chanoclavine-I-17-T	*N*-Demethylchanoclavine-I-17-T
Radioactivity fed	3.41 x 10^6 dpm	3.68 x 10^6 dpm
Amount of alkaloid formed	46 mg	50.5 mg
Total radioactivity of elymoclavine	1.47 x 10^5 dpm	3.44 x 10^3 dpm
Incorporation	4.3%	0.1% (max.)

after 4 recrystallizations had a constant specific radioactivity corresponding to 4.3% incorporation. This is as expected on the basis of earlier findings (10) that one half of the tritium from C–17 of chanoclavine-I is incorporated into C–7 of elymoclavine. In the experiment with *N*-demethylchanoclavine-I-17-T, the specific radioactivity of elymoclavine was not constant after 4 recrystallizations, when the material was used up. At that point it corresponded to an incorporation of less than 0.1%, indicating that *N*-demethylchanoclavine-I-17-T was not converted into the tetracyclic ergolines. Another feeding experiment produced similar results. Since the negative outcome of a feeding experiment with intact cells may simply be due to impermeability of the cell membrane to the substrate, we carried out an uptake experiment. A culture was incubated with *N*-demethylchanoclavine-I-17-T and at several times portions of the mycelium were carefully washed and assayed for radioactivity. This experiment showed that after 2 hours 3.6% of the added substrate was located in the mycelium, which is close to the calculated value (3.9%) for complete equilibration between intracellular and extracellular fluid.

DISCUSSION

The work reported here shows that a new minor alkaloid isolated from *Claviceps* strain SD 58 is the *N*-demethyl analog of (−)-chanoclavine-II, which itself has been isolated before from rye ergot (13) as well as from *Claviceps* strain SD 58 (1). This finding raises the question as to where this and possibly other norchanoclavines stand in the overall picture of ergoline biosynthesis, i.e.,

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are such norchanoclavines precursors of the chanoclavines or are they derived from them by demethylation? There is considerable evidence that *Claviceps* is capable of demethylating *N*-methylated bases in the tryptophan and clavine series (6, 14) and, in fact, a few *N*-nor derivatives of clavine alkaloids have been obtained from *Claviceps* or related fungi (15, 16). These demethylations seem to be fairly nonspecific and are carried out by such enzymes as peroxidase (3, 17). The ability of *Claviceps* to methylate specific compounds of this type, on the other hand, is less well documented (18), but the biosynthesis of ergolines must, of course, involve an *N*-methylation at some stage (5). On mechanistic grounds we had assumed that this methylation takes place at the norchanoclavine stage (1). However, the feeding experiment with *N*-demethylchanoclavine-I-17-T does not seem to bear this out since this compound was not incorporated into elymoclavine Although as a rule negative results of feeding experiments have to be interpreted with caution, there is good reason to believe that this finding is significant and indicates that *N*-demethylchanoclavine-I is not an intermediate in ergoline biosynthesis. Most other possible explanations for the negative outcome of the feeding experiment can be excluded. For example, failure of the precursor to enter the cells can be ruled out on the basis of the uptake experiment. Alkaloid was actively synthesized during the time of the feeding experiment, as evidenced by the incorporation of chanoclavine-I-17-T in the parallel culture. Loss of the tritium label during or prior to the methylation is not only highly unlikely, but can also be excluded because one atom of deuterium from mevalonic acid-3'-D_3 is incorporated into elymoclavine (M. Tcheng-Lin, C. -j. Chang and H. G. Floss, unpublished). We thus conclude that the methylation of the amino nitrogen in the biosynthesis of ergoline alkaloids does not take place at the stage of *N*-demethylchanoclavine-I but either prior to the formation of ring C or at the stage of an as yet unknown product of C ring formation. By analogy it seems probable that the *N*-demethylchanoclavine-II isolated in this study represents not a precursor but a demethylation product of chanoclavine-II.

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TI Alkaloids from the fungus Penicillium aurantio-virens Biourge and some aspects of their formation
AU Solov'eva, T. F.; Kuvichkina, T. N.; Baskunov, B. P.; Kozlovskii, A. G.
CS Inst. of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushkino, 142292, Russia
SO Mikrobiologiya (1995), 64(5), 645-650

TI Alkaloids of Stipa robusta (sleepygrass) infected with an Acremonium endophyte
AU Petroski, Richard J.; Powell, Richard G.; Clay, Keith
CS Natl. Cent. Agric. Util. Res., Agric. Res. Serv., Peoria, IL, 61604, USA
SO Nat. Toxins (1992), 1(2), 84-8

TI Alkaloid composition of Penicillium palitans and Penicillium oxalicum
AU Vinokurova, N. G.; Reshetilova, T. A.; Adanin, V. M.; Kozlovskii, A. G.
CS Inst. Biochem. Physiol. Microorg., Pushchino, USSR
SO Prikl. Biokhim. Mikrobiol. (1991), 27(6), 850-5

TI Enzymic transformation of chanoclavine-I by Penicillium sizovae F-209 cell-free extract
AU Markelova, N. Yu.; Kozlovskii, A. G.
CS Inst. Biochem. Physiol. Microorg., Pushchnio, USSR
SO Prikl. Biokhim. Mikrobiol. (1990), 26(3), 355-9

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AU BACON C W; LYONS P C; PORTER J K; ROBBINS J D
CS TOXICOL. BIOL. CONSTIT. RES. UNIT, R.B. RUSSELL AGRIC. RES. CENT., USDA-ARS, ATHENS, GA.
SO AGRON J, (1986) 78 (1), 106-116.

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AU Cassady, John M.; Abou-Chaar, Charles I.; Floss, Heinz G.
CS Dep. Med. Chem. Pharmacogn., Purdue Univ., Lafayette, Indiana, USA
SO Lloydia (1973), 36(4), 390-6

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AU Erge, D.; Maier, W.; Groeger, D.
CS Inst. Biochem. Pflanzen, Halle/Saale, E. Ger.
SO Biochem. Physiol. Pflanz. (1973), 164(3), 234-47

TI Production of alkaloids and related substances by fungi. III. Isolation of chanoclavine I and two new interconvertible alkaloids, regulovasine A and B, from Penicillium cultures
AU Abe, Matazo; Ohmomo, Sadahiro; Ohashi, Tsutomu; Tabuchi, Takeshi
CS Tokyo Univ. Educ., Tokyo, Japan
SO Nippon Nogei Kagaku Kaishi (1969), 43(8), 575-82
CODEN: NNKKAA
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Н.Г. ВИНОКУРОВА, Т.А. РЕШЕТИЛОВА, В.М. АДАНИН, А.Г. КОЗЛОВСКИЙ

ИССЛЕДОВАНИЕ АЛКАЛОИДНОГО СОСТАВА ГРИБОВ *PENICILLIUM PALITANS* И *PENICILLIUM OXALICUM*

Установлена способность грибов *Penicillium palitans* и *Penicillium oxalicum* синтезировать клавиновые алкалоиды: фумигаклавин А, фумигаклавин В, пироклавин, фестуклавин, ханоклавин-1, а также дипептидный алкалоид пролилвалилдикетопиперазин и α-циклопиазоновую кислоту. Максимальная продуктивность обеих культур наблюдалась при росте на среде с соевой мукой при поверхностном выращивании.

Культуры *Penicillium palitans* и *Penicillium oxalicum* относятся к наиболее известным контаминантам (загрязнителям) пищевых продуктов [1], представляющих в связи с этим потенциальную опасность для здоровья человека и животных. Однако состав микотоксинов у этих грибов исследован недостаточно полно. Имеются разобщенные, отрывочные сведения о способности различных штаммов этих видов синтезировать некоторые микотоксины, в том числе азотсодержащие, относящиеся к алкалоидам. У некоторых штаммов *P. palitans* обнаружены пенитремы А, В и С [2], виридикатин [3]; отдельные штаммы *P. oxalicum* известны как продуценты дикетопиперазиновых алкалоидов оксалина и рокефортина [4].

В работе исследовали состав азотсодержащих микотоксинов, относящихся к алкалоидам, у *P. palitans* ВКМ F-3088 и *P. oxalicum* ВКМ F-478.

ЭКСПЕРИМЕНТАЛЬНАЯ ЧАСТЬ

Методика

Засев культур *Penicillium palitans* ВКМ F-3088 и *Penicillium oxalicum* ВКМ F-478 проводили спорами и выращивали либо глубинным способом в качалочных колбах объемом 750 мл со 150 мл среды при 24° и 180—200 об/мин, либо стационарно в таких же колбах.

Для выращивания использовали синтетическую среду Абе [5], комплексную среду Чапека — Докса (г/л: сахароза — 30, $NaNO_3$ — 2, K_2HPO_4 — 1, $MgSO_4$ — 0,5, $FeSO_4$ — 0,01, дрожжевой экстракт — 5,1; pH 7,3), комплексную среду с соевой мукой (г/л: глюкоза — 50, пептон — 10, соевая мука — 5, KNO_3 — 2, $MgSO_4 \cdot 7H_2O$ — 0,5; $CaCl_2$ — 0,1; pH 6,2).

Выделение алкалоидов из фильтрата культуральной жидкости и из мицелия проводили экстракцией хлороформом и винной кислотой, как описано ранее [6]. α-Циклопиазоновую кислоту (α-ЦПК) выделяли по методике [7], очищали препаративной тонкослойной хроматографией (ТСХ) в системе I: этилацетат: MeOH : 25%-ный NH_4OH (85:15:10) и элюировали с носителя этанолом.

Оценку содержания эргоалкалоидов проводили спектрофотометрически, используя коэффициент молярной экстинкции фестуклавина [8]. Количество α-ЦПК определяли при 284 нм [9].

Идентификацию соединений проводили по их хроматографической подвижности на пластинках силуфола УФ-254 «Kavalier» (ЧССР), проявляя в системе $CHCl_3$: MeOH : 25%-ный NH_4OH (90:10:0,5 — II, 90:10:0,3 — III, 90:10:0,1 — IV, 80:20:0,2 — V). Разделенные вещества обнаруживали в виде поглощающих под УФ-светом пятен или в виде окрашенных зон после опрыскивания реактивом Эрлиха.

АДАНИН, А.Г. КОЗЛОВСКИЙ

ГО СОСТАВА ГРИБОВ *ICILLIUM OXALICUM*

nicillium palitans и *Penicillium* оиды: фумигаклавин А, фуми-ноклавин-1, а также дипептид-ин и α-циклопиазоновую кисло-их культур наблюдалась при кностном выращивании.

n oxalicum относятся к наиболее ищевых продуктов [1], представля-для здоровья человека и животных. в исследован недостаточно полно. о способности различных штаммов сины, в том числе азотсодержащие, штаммов *P. palitans* обнаружены льные штаммы *P. oxalicum* известны оидов оксалина и рокефортина [4]. ащих микотоксинов, относящихся *xalicum* ВКМ F-478.

АСТЬ

F-3088 и *Penicillium oxalicum* щивали либо глубинным способом мл среды при 24° и 180—200 об/мин,

ескую среду Абе [5], комплексную NO_3 — 2, K_2HPO_4 — 1, $MgSO_4$ — 0,5, ,3), комплексную среду с соевой мукой — 5, KNO_3 — 2, $MgSO_4 \cdot 7H_2O$ — 0,5;

ьтуральной жидкости и из мицелия ой кислотой, как описано ранее [6]. деляли по методике [7], очищали ей (ТСХ) в системе I: этилацетат: вали с носителя этанолом. или спектрофотометрически, исполь-стуклавина [8]. Количество α-ЦПК

их хроматографической подвижности er» (ЧССР), проявляя в системе — II, 90:10:0,3 — III, 90:10:0,1 — IV, наруживали в виде поглощающих к зон после опрыскивания реактивом

Для получения УФ- и масс-спектров индивидуальные алкалоиды выделяли методом препаративной тонкослойной хроматографии на пластинках силуфола. Вещество с носителя элюировали смесью растворителя $CHCl_3$: MeOH (1:1).

Спектры поглощения алкалоидов в УФ-области регистрировали на спектрофотометре «Beckman DV8V» (США).

Масс-спектры метаболитов получены на масс-спектрометре «Finnigan MAT 8430» (ФРГ) с использованием прямого ввода образца в область ионизации. Температура источника ионов 250°; температура испарения образцов 50—200°.

Аминокислотный состав дипептида определяли на анализаторе аминокислот «Biotronik LC6000E» (ФРГ). ИК-спектры образцов снимали на спектрометре FTIRR 1710 «Perkin-Elmer» (США).

Использованные в работе в качестве свидетелей фестуклавин и изофумигаклавины А и В выделены ранее из культуры *P. roqueforti* [10]. Ханоклавин-1 и агроклавин получены из культуральной жидкости *Claviceps* sp., ИБФМ-F-401 [11], костаклавин и эпикостаклавин — из культуры *P. gorlenkoanum* ИБФМ F-201 [6], α-ЦПК — из *P. cyclopium* [12].

Фумигаклавины А и В выделяли из фильтрата культуральной жидкости (10 л) 27-суточной культуры *P. palitans*, выращенной при поверхностном культивировании на среде с соевой мукой. Алкалоиды извлекали экстракцией хлороформом [6]. Предварительное деление смеси (около 200 мг) на отдельные компоненты осуществляли хроматографически на колонке 2,6 × 24 мм, заполненной сефадексом LH-20 (60 г). В качестве элюента использовали смесь растворителей хлороформ:гексан (6:4). Контроль за разделением проводили методом ТСХ в системе III. Фракции, содержащие фумигаклавины, упаривали и подвергали дальнейшей очистке на стеклянных пластинках с силикагелем LSL 5/40 мкм (ЧССР) в системе II. Выделенные фумигаклавины А и В перекристаллизовывали из водного метанола.

Результаты и их обсуждение

Известно, что культура *P. palitans* NRRL 3468, выделенная из коммерческого корма, явилась причиной смерти животных в результате отравления треморгенными микотоксинами [2,3]. Из мицелия гриба, выращенного на среде Чапека — Докса с дрожжевым экстрактом (0,5%) в стационарных условиях, были выделены тремортины А, В, С и виридикатин. Изучаемая нами культура гриба *P. palitans* ВКМ-3088, выращенная в аналогичных условиях, не продуцировала сколько-нибудь заметных количеств указанных микотоксинов, но обнаружила способность синтезировать клавиновые алкалоиды.

Для выделения алкалоидов использовали 15-суточный мицелий, выращенный в стационарных условиях на среде с соевой мукой. Предварительными опытами установлено, что именно в этих условиях накапливалось наибольшее количество алкалоидов.

По данным ТСХ, экстракт из разрушенного дезинтеграцией мицелия содержал в основном семь метаболитов, дающих специфическую окраску с реактивом Эрлиха (рис. 1). При разделении смеси на отдельные компоненты методом препаративной ТСХ в системе V было обнаружено еще одно соединение алкалоидной природы, присутствующее в экстракте в значительно меньших количествах, чем основные компоненты (на хроматограмме оно расположено между 2-м и 3-м соединениями). Окончательную очистку алкалоидов осуществляли повторной препаративной хроматографией в системах II—V.

На основании физико-химических характеристик и хроматографической подвижности выделенные метаболиты были идентифицированы как фумигаклавин А (1), пролилвалилдикетопиперазин (2), агроклавин (3а), пироклавин (3), фестуклавин (4), фумигаклавин В (5), α-ЦПК (6) и ханоклавин-1 (7) (табл. 1).

Полученные для этих соединений масс-спектры (табл. 1) и УФ-спектры соответствуют имеющимся в литературе [8—10,13]. Сохроматография фестуклави-

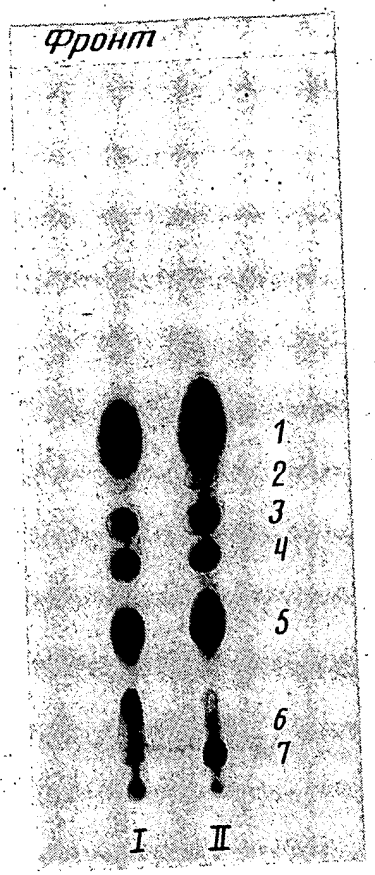

Рис. 1

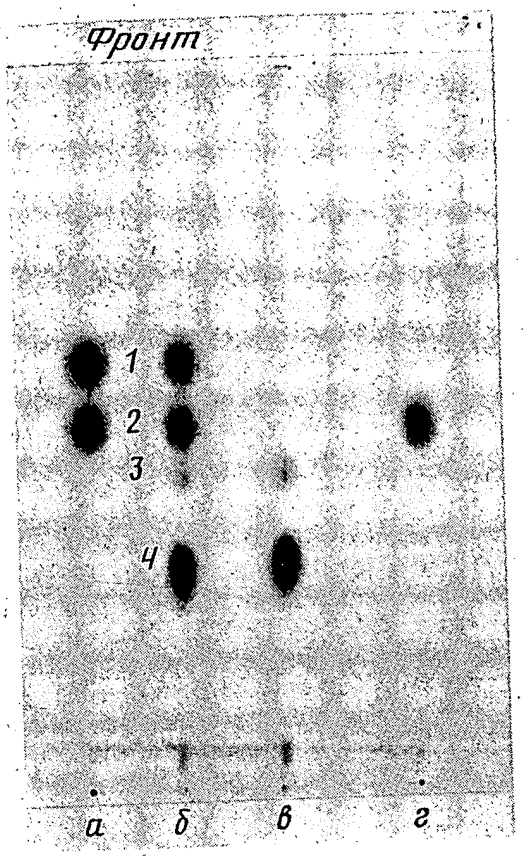

Рис. 2

Рис. 1. ТСХ смеси алкалоидов из мицелия грибов *P. palitans* (I) и *P. oxalicum* (II), выращенных на комплексной среде с соевой мукой при поверхностном культивировании. Система $CHCl_3$: MeOH : 25%-ный NH_4OH (90:10:0,3) : *1* — фумигаклавин А, *2* — пролилвалилдикетопиперазин, *3* — пироклавин, *4* — фестуклавин, *5* — фумигаклавин В; *6* — α-ЦПК, *7* — ханоклавин-I

Рис. 2. ТСХ изомерных клавиновых алкалоидов с m/z 240 в системе $CHCl_3$: MeOH : 25%-ный NH_4OH (80:20:0,2): *1* — пироклавин, *2* — фестуклавин, *3* — эпикостаклавин, *4* — костаклавин, *а* — пироклавин + фестуклавин, *б* — смесь изомерных алкалоидов, *в* — эпикостаклавин + костаклавин, *г* — фестуклавин

на, ханоклавина-1, агроклавина в системах II—V и α-ЦПК в системе I с заведомо известными образцами подтвердила идентичность этих соединений.

Хроматографическая подвижность соединений 1 и 5, масс-спектры которых соответствовали изофумигаклавинам А и В, обнаруженным ранее у *P. roqueforti* ИБФМ F-141 [10], отличалась от хроматографической подвижности изофумигаклавинов и соответствовала хроматографической подвижности изомерных фумигаклавинов А и В [14]. ИК-спектры полностью совпадали с ИК-спектрами, приведенными в литературе для этих соединений [9]. Температура плавления выделенного фумигаклавина А — 80—82° (лит. 84—85°), фумигаклавина В — 238—240° (лит. 244—245) [8]. Хроматографическая подвижность соединения 3 с мол. массой 240 отличалась от хроматографической подвижности конформационных изомеров фестуклавина, костаклавина и эпикостаклавина (рис. 2) и соответствовала приведенной в литературе для пироклавина [14].

Соединение 2, несмотря на то, что с реактивом Эрлиха давало бледно-голубое окрашивание, не обладало заметным поглощением в УФ-диапазоне

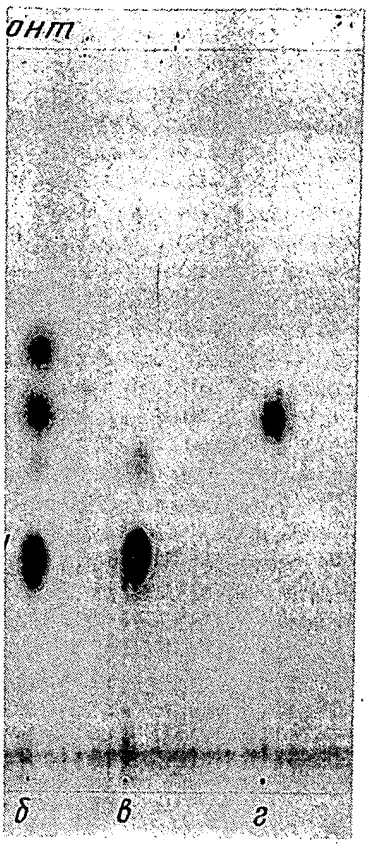

Рис. 2

tans (I) и *P. oxalicum* (II), выращенных ностном культивировании. Система аклавин А, *2* — пролилвалилдикетопи- лавин В; *6* — α-ЦПК, *7* — ханоклавин-I

40 в системе $CHCl_3$: MeOH : 25%-ный — эпикостаклавин, *4* — костаклавин, ноидов, *в* — эпикостаклавин + костакла-

и α-ЦПК в системе I с заведомо ь этих соединений.

й 1 и 5, масс-спектры которых ружденным ранее у *P. roqueforti* ской подвижности изофумигакла- подвижности изомерных фуми- ью совпадали с ИК-спектрами ний [9]. Температура плавления . 84—85°), фумигаклавина В — подвижность соединения 3 с мол. подвижности конформационных лавина (рис. 2) и соответствовала

тивом Эрлиха давало бледно- поглощением в УФ-диапазоне

Таблица 1

Масс-спектры метаболитов *P. palitans* 3088

№	Метаболит	*m/z* (относительная интенсивность, %)	Литературный источник
1	Фумигаклавин А	127(13), 144(31), 154(68), 155(20), 167(29), 168(22), 180(35), 195(25), 207(13), 223(17), 237(47), 239(100), 255(18), 298(43, M^+), 299(9)	[9, 19]
2	Пролилвалилдикетопиперазин	39(14), 41(30), 43(17), 55(18), 70(100), 72(28), 97(9), 110(8), 125(25), 138(6), 154(80), 155(8), 194(14), 196(4, M^+)	[15]
3а	Агроклавин	127(5), 140(5), 154(9), 155(4), 167(8), 181(1), 192(5), 196(7), 223(11), 237(100), 238(65, M^+), 239(11)	[20]
3	Пироклавин	127(9), 144(22), 154(17), 155(6), 167(13), 168(10), 181(5), 182(8), 183(6), 197(23), 198(5), 223(7), 225(10), 239(15), 240(100, M^+), 241(18)	[20]
4	Фестуклавин	127(8), 144(22), 154(15), 155(6), 167(10), 168(9), 181(5), 182(9), 183(6), 197(21), 198(5), 223(5), 225(9), 239(14), 240(100, M^+), 241(18)	[10]
5	Фумигаклавин В	127(25), 140(13), 144(22), 154(72), 155(26), 167(18), 168(33), 181(17), 182(16), 192(7), 196(13), 197(16), 210(20), 213(10), 223(10), 239(85), 240(16), 255(15), 256(100, M^+), 259(19)	[9]
6	Ханоклавин-1	144(12), 154(81), 155(58), 167(73), 168(55), 196(19), 206(14), 223(11), 237(47), 238(23), 256(53, M^+), 257(10)	[20]
7	α-Циклопиазоновая кислота	70(46), 127(16), 154(63), 155(41), 167(13), 181(57), 182(100), 196(55), 336(75, M^+), 337(19)	[9]

(220—350 нм), характерном для эргоалкалоидов. В масс-спектре метаболита 2 (табл. 1) наряду с пиком молекулярного иона *m/z* 196 присутствовали интенсивные пики с *m/z* 154 и 70, характерные для дикетопиперазинов, содержащих пролин [15]. По данным аминокислотного анализа, в состав метаболита входят две аминокислоты — пролин и валин. В ИК-спектре, снятом в KBr, обнаружена полоса поглощения при 3210 $см^{-1}$, характерная для валентных колебаний NH-группы вторичного амида. Интенсивные полосы поглощения при 1675 и 1650 (плечо) $см^{-1}$ указывают на присутствие карбонильной группы амида. Изложенное дало основание считать, что метаболит 2 является пролилвалилдикетопиперазином, структурная формула которого приведена на рис. 3. Аналогичное соединение было выделено из культуральной жидкости *Streptomyces* sp. S-580 и *Rosellinia necatrix* [16]. Подобные дикетопиперазины обнаружены у целого ряда микроорганизмов, в том числе у грибов рода *Penicillium* и *Aspergillus* [17].

В процессе работы по исследованию алкалоидообразования у различных пенициллов была выявлена культура *P. oxalicum* ВКМ F-478 с алкалоидным составом, аналогичным *P. palitans*. Сохроматографией индивидуальных метаболитов, выделенных из мицелия этих продуцентов, была установлена идентичность таких алкалоидов, как фумигаклавин А, пролилвалилдикетопиперазин, пироклавин, фестуклавин, фумигаклавин В, ханоклавин-1 и α-ЦПК. Наличие этих соединений подтверждено УФ-спектроскопией и масс-спектрометрией.

Отмечены различия в соотношении отдельных компонентов алкалоидных фракций изучаемых культур. Так, содержание ханоклавина-1 в мицелии *P. oxalicum* было значительно выше, чем у *P. palitans* (рис. 1). В отношении α-ЦПК наблюдается обратная картина. Основное количество кислоты у *P. oxalicum* было сосредоточено в мицелии и не превышало 0,05 мг/г сухой биомассы. *P. palitans* синтезирует значительно большее количество α-ЦПК. Содержание ее в культуральной жидкости постепенно повышалось в процессе культивирования и к 26-м сут достигало 2 мг/л. Концентрация внутриклеточной α-ЦПК составляла около 0,3 мг/г сухой биомассы.

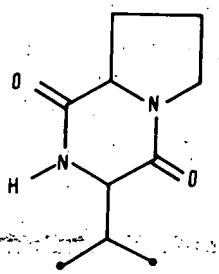

Рис. 3. Структура пролилвалилдикетопиперазина

Таким образом, можно сделать заключение об аналогичности алкалоидного состава двух различных видов грибов — *P. palitans* ВКМ F-3088 и *P.oxalicum* ВКМ F-478; при этом следует отметить различие в соотношении отдельных компонентов алкалоидных фракций.

Известно, что алкалоидообразование у грибов в значительной степени зависит от среды и способа культивирования [18]. В связи с этим нами исследована способность обеих культур синтезировать алкалоиды на синтетической и комплексной средах при глубинном и поверхностном способах выращивания. Во многих работах по исследованию биосинтеза клавиновых и дикетопиперазиновых алкалоидов у грибов рода *Penicillium* в качестве контрольной использовали среду Абе, содержащую маннит и янтарную кислоту [18]. Эта среда, как правило, обеспечивает хороший рост и алкалоидообразование. Однако в нашем случае, несмотря на хороший рост культур, алкалоидообразование на этой среде выражено крайне слабо. В культуральной жидкости выявлены незначительные количества фумигаклавинов А и В, в мицелии алкалоиды не обнаруживались.

Таблица 2

Количество биомассы и содержание алкалоидов в культуральной жидкости и мицелии *P. palitans* и *P. oxalicum* при поверхностном и глубинном культивировании в различных средах

Среда	Способ культивирования	Количество биомассы, г/л		Содержание алкалоидов			
				в культуральной жидкости, мг/л		в мицелии, мг/г сухой биомассы	
		P. palitans	*P. oxalicum*	*P. palitans*	*P. oxalicum*	*P. palitans*	*P. oxalicum*
Абе	Глубинный	9,1	17,0	1,0	1,5	0	0
Чапека—Докса	Глубинный	7,7	6,8	0	Сл.	0	Сл.
	Поверхностный	13,3	12,5	2,3	4,0	0,1	0,4
С соевой мукой	Глубинный	24,7	17,6	Сл.	0,7	Сл.	0,1
	Поверхностный	19,4	15,2	12,0	20,0	0,7	1,6

Практически не наблюдалось ал
богатых питательных средах п
поверхностном выращивании инт
отмечено на среде с соевой мукой

Общность изучаемых культур
и экскреции алкалоидов в среду
Установлено, что при росте на
алкалоидных фракций, выделе
фумигаклавины А и В (а такж
и пироклавин слабо экскретиру
мицелии. Авторы выражают бла
тров и консультацию при их инте

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Институт биохимии и физиологии микроорганизмов АН СССР, Пущино

N.G. VINOKUROVA, T.A. R

STUDY OF THE ALKALOID
AND *P*

Institute of Biochemistry and Physiolog

The fungi *Penicillium palitans* an
alkaloids fumigaclavine A, fumiga
as well as a prolylvalyl-diketopip
The highest productivity of the tw
a medium with soya flour.

Практически не наблюдалось алкалоидообразование у грибов, выращенных на богатых питательных средах при глубинном способе культивирования. При поверхностном выращивании интенсивное алкалоидообразование у обеих культур отмечено на среде с соевой мукой (табл. 2).

Общность изучаемых культур заключается также и в характере накопления и экскреции алкалоидов в среду при культивировании в стационарных условиях. Установлено, что при росте на комплексных средах основными компонентами алкалоидных фракций, выделенных из культуральной жидкости, являются фумигаклавины А и В (а также ханоклавин-1 для *P. oxalicum*). Фестуклавин и пироклавин слабо экскретируются в среду и накапливаются в основном в мицелии. Авторы выражают благодарность М.Ю. Нефедовой за снятие ИК-спектров и консультацию при их интерпретации.

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STUDY OF THE ALKALOID COMPOSITION OF *PENICILLIUM PALITANS* AND *PENICILLIUM OXALICUM*

Institute of Biochemistry and Physiology of Microorganisms, USSR Academy of Sciences, Pushchino

The fungi *Penicillium palitans* and *Penicillium oxalicum* are able to synthesize clavine alkaloids fumigaclavine A, fumigaclavine B, pyroclavine, festuclavine, chanoclavine-1 as well as a prolylvalyl-diketopiperazine cyclic dipeptide and α-cyclopiasonic acid. The highest productivity of the two cultures was observed upon surface cultivation on a medium with soya flour.

SO J GEN MICROBIOL, (1979) 113 (1), 119-126.

Vol 10

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AU Maier, W.; Groeger, D.
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The alkaloids of corn ergot
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SO Planta Med. (1963), 11(4), 392-8
DT

54:5724f-g
TI Ergot alkaloids.
AU Voigt, R.
CS Humboldt-Univ., Berlin
SO Naturwissenschaften (1959), 46, 401
DT

Irene Marx
Art Unit 1651
CMI 10-E-05,
Mail Box 11-B-01
703-308-2922

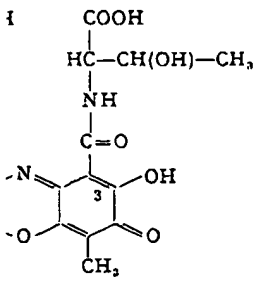
09824590

7/17

CAI 7/19

L. C WOS
7-18-RC

iprodukt konnten wir durch Vergleich mit hen Präparat als Desamino-actinocinyl-bis-lentifizieren (Schmp. Misch-Schmp., Absorp-R-Spektrum, R_F-Wert).



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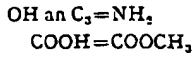


II

us Actinocinyl-bis-[L-threoninmethylester] diges Kochen mit 50%iger Essigsäure und rseifung mit verdünntem Alkali (so modi-romophor intakt bleibt) gewonnen. Orange-n Schmp. 214° Zers. (Berl-Block, korrig.) °, in Methanol. $C_{24}H_{25}N_3O_{11}$ (531,5) Ber. N 7,91; Gef. C 54,26, H 5,03, N 7,98.

Abbau- und Trennungsverfahren läßt sich ycinen 1. anders als bisher[2]) die Konstitution ermitteln und 2. feststellen, ob die Carboxy-mophors mit Threonin verknüpft sind.

misches Institut der Universität, Göttingen

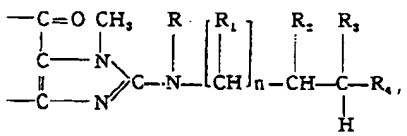
s Brockmann und Hans-Sieghard Petras

m 15. April 1959

H., u. H. Gröne: Chem. Ber. 87, 1036 (1954). — u. K. Vohwinkel: Chem. Ber. 89, 1337 (1956).

r Coffeinderivate mit geringerer Giftwirkung id stärkerer Wirkung als Coffein

allgemein als ein ungiftiges Genußmittel. ebungswirkung im Vordergrund pharmako-Anwendung steht, sind doch die Herz-e nach übermäßigem und andauerndem treten, sehr unangenehm. Es sind bereits he[1–5]) unternommen worden, Derivate auf-n entweder die Herzwirkung des Coffeins igswirkung des Coffeins bei verminderter l verschoben sind. Diese experimentellen sher nicht zum Erfolg führen können; es war igen, ein für die Therapie brauchbares ver-ivat aufzubauen, welches etwa mit den ver-des Morphins oder anderer Wirkstoffe des lichen werden könnte. Nach der Synthese[6]) von Theophyllin- und Coffeinderivaten hat-offein-(8)-alkanolaminen der nachfolgenden citution



$-CH_3$, $-C_2H_5$, allgemein Alkyle mit 1 bis H oder Alkyle, R_2=H, OH, Alkyle oder yle, $-OH$, $-O-CH_3$, $-O-C_2H_5$, R_4=H 1 bis 5 C-Atomen oder Phenyl, n = 0 oder 1 örperklasse gefunden, die drei- bis sechsmal l dazu pharmakodynamisch stärker wirksam

lieser Substanzen erfolgte durch Umsetzung Schmp.: 162 bis 164° C, γ-N-[Coffeino-(8)]-amino-propanol-(3), Schmp.: 224 bis 226° C.

Wissenschaftliches Privat-Forschungslabor, Berlin-Zehlendorf, Jänickestraße 13

Josef Klosa (Berlin) und Hans Starke (Dresden)

Eingegangen am 13. April 1959

[1]) Fischer, E.: Liebigs Ann. Chem. 215, 283 (1882). — [2]) Cramer, L.: Ber. dtsch. chem. Ges. 27, 9098 (1894). — [3]) Einhorn, A., u. E. Baumeister: Ber. dtsch. chem. Ges. 31, 1138 (1898). — [4]) Gomberg, J.: Amer. Chem. J. 23, 51 (1901). — [5]) Blicke, F. F., u. H. C. Godt jr.: J. Amer. Chem. Soc. 76, 2835 (1954). — [6]) Klosa, J.: 5. u. 6. Mitt. über Synthesen in der Theophyllinreihe. J. prakt. Chem. 6, 182, 187 (1958). — [7]) Zahlreiche weitere Substanzen sind die Grundlage einer Anzahl von Patentanmeldungen in Deutschland und dem Auslande.

Beitrag zur Kenntnis von Mutterkornalkaloiden

Aus deutschem Roggenmutterkorn konnten wir — wie kürzlich berichtet[1]) — ein Alkaloid isolieren und auf dessen etwaige Identität mit dem aus japanischem Gräsermutterkorn von Abe und Yamatodani[2]) gewonnenen Secaclavin (Alkaloid X) hinweisen. Nach neuesten Angaben von Abe[3]) bestand jedoch die Möglichkeit, daß Secaclavin mit Chanoclavin, einem von Hofmann, Brunner, Kobel und Brack[4]) aus saprophytischen Kulturen des Mutterkornpilzes von afrikanischer Kolbenhirse isolierten und in seiner Struktur aufgeklärten Alkaloid, identisch ist. Secaclavin (Alkaloid X)*), Chanoclavin*) und die von uns abgetrennte Substanz wurden daher papierchromatographisch überprüft. Hierzu fanden die Lösungsmittelsysteme Butanol-Eisessig-Wasser 4:1:5, Butanol-Pyridin-Wasser 4:1:5, Äthylacetat-Wasser 1:1 (jeweils auf unbehandeltem Papier), Äther-Aceton-Wasser 2:4:2 (Papier mit 0,2%iger Weinsäurelösung getränkt), wassergesättigtes Chloroform (p_H 7-gepuffertes Papier) und Chloroform (formamidgetränktes Papier) Anwendung. Die genannten Verbindungen wiesen gleiche *Rf*-Werte auf. Bei Mischchromatogrammen ließ sich gleichfalls lediglich ein Fleck nachweisen. Die Identität von Chanoclavin, Secaclavin und dem von uns aufgefundenen Alkaloid erscheint somit gesichert.

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Pharmazeutisches Institut der Humboldt-Universität, Berlin (Direktor: Prof. Dr. F. Weiss)

R. Voigt

Eingegangen am 26. April 1959

*) Herrn Dr. M. Abe, Osaka, und Herrn Dr. A. Hofmann, Basel, sind wir für die freundliche Überlassung von Reinsubstanz zu außerordentlichem Dank verpflichtet.

[1]) Voigt, R.: Naturwiss. 46, 77 (1959). — [2]) Abe, M., u. T. Yamatodani: J. Agric. Chem. Soc. Japan 28, 501 (1954). — [3]) Abe, M.: Privatmitteilung. — [4]) Hofmann, A., R. Brunner, H. Kobel u. A. Brack: Helv. chim. Acta 40, 1358 (1957).

Kaempferol from Flowers of Ervatamia Coronaria Stapf

On account of its immense therapeutic value[1]) Ervatamia Stapf., Syn., Tabernaemontana R. Br. (Apocynaceae) early attracted attention. Although the milky latex, the bark and the root of the genus have been extensively examined for various plant products, no mention so far appears to have been made of the presence of flavonoids. The isolation of a crystalline flavonoid compound from the flowers of the species is now reported.

The ethanol extract on purification by suitable solvent fractionation, and subsequent treatment with neutral and

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TI Alkaloids from the fungus Penicillium aurantio-virens Biourge and some aspects of their formation
AU Solov'eva, T. F.; Kuvichkina, T. N.; Baskunov, B. P.; Kozlovskii, A. G.
CS Inst. of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushkino, 142292, Russia
SO Mikrobiologiya (1995), 64(5), 645-650

TI Alkaloids of Stipa robusta (sleepygrass) infected with an Acremonium endophyte
AU Petroski, Richard J.; Powell, Richard G.; Clay, Keith
CS Natl. Cent. Agric. Util. Res., Agric. Res. Serv., Peoria, IL, 61604, USA
SO Nat. Toxins (1992), 1(2), 84-8

TI Alkaloid composition of Penicillium palitans and Penicillium oxalicum
AU Vinokurova, N. G.; Reshetilova, T. A.; Adanin, V. M.; Kozlovskii, A. G.
CS Inst. Biochem. Physiol. Microorg., Pushchino, USSR
SO Prikl. Biokhim. Mikrobiol. (1991), 27(6), 850-5

TI Enzymic transformation of chanoclavine-I by Penicillium sizovae F-209 cell-free extract
AU Markelova, N. Yu.; Kozlovskii, A. G.
CS Inst. Biochem. Physiol. Microorg., Pushchnio, USSR
SO Prikl. Biokhim. Mikrobiol. (1990), 26(3), 355-9

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AU BACON C W; LYONS P C; PORTER J K; ROBBINS J D
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SO AGRON J, (1986) 78 (1), 106-116.

Ergot alkaloids. Isolation of N-demethylchanoclavine-II from Claviceps strain SD 58 and the role of demethylchanoclavines in ergoline biosynthesis
AU Cassady, John M.; Abou-Chaar, Charles I.; Floss, Heinz G.
CS Dep. Med. Chem. Pharmacogn., Purdue Univ., Lafayette, Indiana, USA
SO Lloydia (1973), 36(4), 390-6

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AU Erge, D.; Maier, W.; Groeger, D.
CS Inst. Biochem. Pflanzen, Halle/Saale, E. Ger.
SO Biochem. Physiol. Pflanz. (1973), 164(3), 234-47

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AU Abe, Matazo; Ohmomo, Sadahiro; Ohashi, Tsutomu; Tabuchi, Takeshi
CS Tokyo Univ. Educ., Tokyo, Japan
SO Nippon Nogei Kagaku Kaishi (1969), 43(8), 575-82
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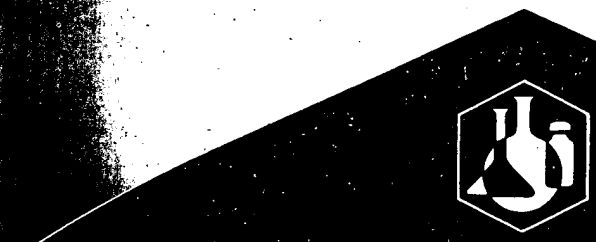

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1990

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**HYDRO-3H-1,4-BENZODIAZEPIN-
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Том 26 1990 ВЫП. 3

УДК 582.282.123.2.017.7

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Н. Ю. МАРКЕЛОВА, А. Г. КОЗЛОВСКИЙ

ФЕРМЕНТАТИВНОЕ ПРЕВРАЩЕНИЕ ХАНОКЛАВИНА I БЕСКЛЕТОЧНЫМ ЭКСТРАКТОМ *PENICILLIUM SIZOVAE* F-209

В бесклеточных экстрактах гриба *Penicillium sizovae* F-209 обнаружена активность ферментативного комплекса, превращающего ханоклавин I. Подобраны оптимальные условия для действия этой ферментной системы: концентрация кофакторов, pH и температура реакционной смеси, при которых достигается около 50% превращения ханоклавина I бесклеточным экстрактом мицелия. Основными продуктами превращения ханоклавина I являются агроклавин I и эпоксиагроклавин I.

Ханоклавин I относится к клавиновым алкалоидам — производным 6,8-диметилэрголина, однако отличается от других клавиновых алкалоидов открытым кольцом D-эрголинового ядра (рис. 1). Ханоклавин I является промежуточным соединением в биогенезе эргоалкалоидов. У грибов рода *Claviceps* достаточно полно установлены пути биосинтеза алкалоидов и выделен ряд ферментов, осуществляющих отдельные этапы их биогенеза [1—4]. У пенициллов, синтезирующих эргоалкалоиды, отличающиеся от алкалоидов *Claviceps* стереохимически, отмечены изменения в общей схеме биосинтеза [5—7]. В связи с этим представляет

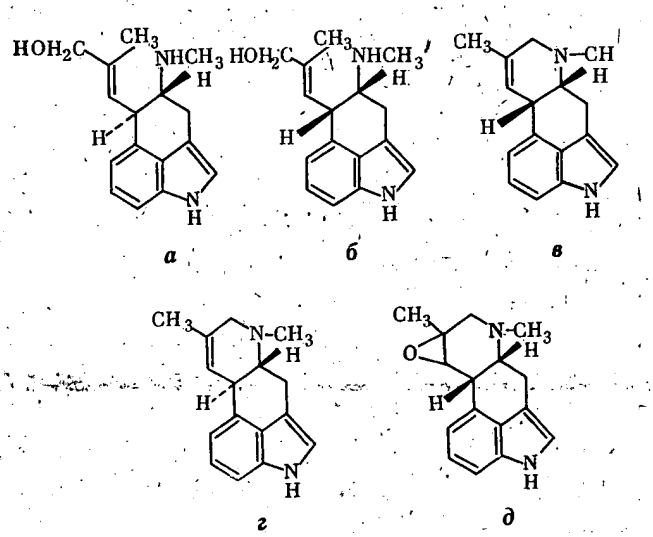


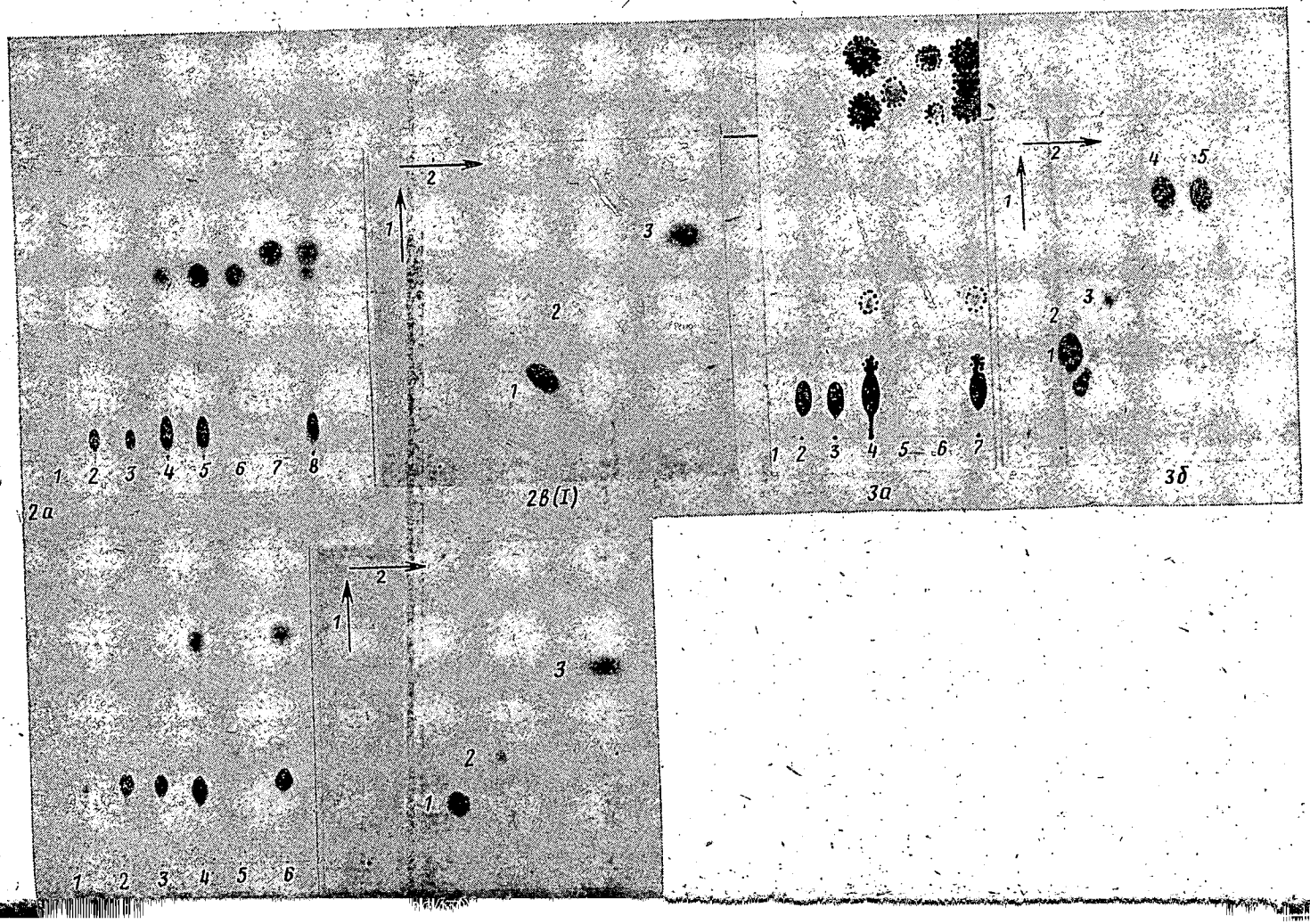
Рис. 1. Структурные формулы ханоклавина I (*а*), ханоклавина III (*б*), агроклавина I (*в*), агроклавина (*г*) и эпоксиагроклавина I (*д*)

интерес изучение биогенеза эргоалкалоидов у грибов рода *Penicillium* и изучение отдельных ферментов или ферментных систем, осуществляющих биосинтез алкалоидов. Настоящее сообщение касается исследования превращения ханоклавина I бесклеточными препаратами из *P. sizovae* F-209.

ЭКСПЕРИМЕНТАЛЬНАЯ ЧАСТЬ

Методика

В работе использовали культуру *Penicillium sizovae* ВКМ F-209. Выращивание проводили на качалке (24° и 220 об/мин) в колбах объемом 750 мл со 150 мл среды следующего состава (г/л дистиллирован-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2б (I)
1 2 3 4 5 6
3а
1 2 3 4 5 6 7
3б
1 2 3 4 5

ной воды): маннит — 50, янтарная кислота — 5,4, $MgSO_4 \cdot 7H_2O$ — 0,3, KH_2PO_4 — 1,0, pH доводили конц. NH_4OH до 5,2. Засев проводили спорами 7-суточной культуры, выращенной на скошенном агаре. 10 мл 3-суточного инокулята переносили в колбы со средой того же состава и проводили дальнейшее выращивание. Определение активности ферментативного комплекса, осуществляющего превращение ханоклавина I, определяли по методу [1] в нашей модификации. Мицелий отфильтровывали, промывали 0,1 М фосфатным буфером (pH 8,0), содержащим 0,4 мл 2-меркаптоэтанола, 100 мг ЭДТА и 10 мл глицерина на 1 л. Для получения бесклеточного экстракта 5 г сырой биомассы разрушали растиранием в жидком азоте, суспендировали в 10 мл того же буфера и центрифугировали (20 000 g, 50 мин, 4°). Супернатант использовали в качестве бесклеточного экстракта. Ферментативную активность определяли следующим образом: реакционную смесь (2 мл), содержащую 0,5 мг ханоклавина I, 2,5 мкМ НАДФН, 10 мкм АТР, 0,5 мл бесклеточного экстракта в 0,1 М фосфатном буфере (pH 8,0) инкубировали 3 ч при 37°. Затем реакцию останавливали, доводя pH конц. NH_4OH до 11—12. Алкалоиды из реакционной смеси экстрагировали дважды этилацетатом (об/об). Экстракт упаривали под вакуумом в водяной бане при 37° и проводили качественный анализ алкалоидов методом ТСХ на силуфоле, проявляя в системах хлороформ — метанол — 25%-ный аммиак (I) 90 : 10 : 0,1 (об/об) и (II) 90 : 10 : 1,5 (об/об) [7].

Алкалоиды обнаруживали в виде поглощающих УФ-пятен и после опрыскивания реактивом Эрлиха в виде окрашенных зон. Идентификацию соединений проводили путем сравнения их R_f со свидетелями и методом сохроматографии со свидетелями. Количественный анализ алкалоидов проводили денситометрически (Chromosoan 3, Joyce Loebl.). Активность исследуемого ферментативного комплекса оценивали по количеству образовавшегося агроклавина-I. Исследовали 3- и 4-суточные культуры *P. sizovae*. Контроль при определении активности проводили со следующими реакционными смесями: 1) кофакторы + буфер + ханоклавин I; 2) буфер + ханоклавин I; 3) кофакторы + буфер + бесклеточный экстракт, подвергнутый 10-минутному кипячению; 4) бесклеточный экстракт + буфер + кофакторы; 5) бесклеточный экстракт + буфер.

Результаты и их обсуждение

При инкубации ханоклавина I с бесклеточным экстрактом из 4-суточной культуры *P. sizovae* в качестве основного продукта реакции образуется только агроклавин I (рис. 2, *а—в*). При действии бесклеточного экстракта из 3-суточной культуры в инкубационной смеси наряду с продуктом превращения ханоклавина I агроклавином I в качестве основного компонента образуется эпоксиагроклавин I, а также небольшие количества ханоклавина III, секоагроклавина и два неидентифицированных соединения с R_f меньше R_f ханоклавина I (рис. 3, *а*, *б*). Бесклеточные экстракты из суточной и 2-суточной культур были неактивными. В контрольных опытах превращения ханоклавина I не было.

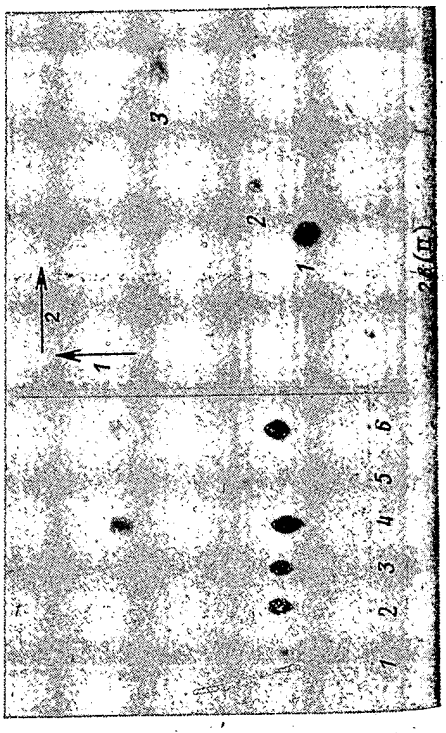

Рис. 2. ТСХ экстрактов инкубационной смеси при превращении ханоклавина I бесклеточным экстрактом из 4-суточной культуры *P. sizovae*. *а* — система I: *1* — контроль 4, *2* — контроль 1, *3* — контроль 2, *4* — опыт, *5* — опыт + агроклавин I, *6* — агроклавин I, *7* — апроклавин, *8* — опыт + агроклавин; *б* — система II: *1* — контроль 4, *2* — контроль 1, *3* — контроль 2, *4* — опыт, *5* — ханоклавин III, *6* — опыт + ханоклавин III, *в* — двумерная хроматограмма. I — опыт, II — опыт + ханоклавин III (I — система II, *2* — система I). *1* — ханоклавин I, *2* — ханоклавин III, *3* — агроклавин I. Обозначения приведенных контролей указаны в главе «Методика»

Рис. 3. ТСХ экстрактов инкубационной смеси при превращении ханоклавина I бесклеточным экстрактом 3-суточной культуры *P. sizovae*. *а* — система I, *1* — контроль 4, *2* — контроль 1, *3* — контроль 2, *4* — опыт, *5* — агроклавин, *6* — агроклавин I + эпоксиагроклавин I, *7* — опыт + агроклавин I + агроклавин + эпоксиагроклавин I; *б* — двумерная хроматограмма. *1* — система II, *2* — система I. *1* — ханоклавин I, *2* — ханоклавин III, *3* — секоагроклавин, *4* — агроклавин I, *5* — эпоксиагроклавин I. Обозначения приведенных контролей указаны в главе «Методика»

Для подбора оптимальных условий превращения ханоклавина I изучали влияние природы кофакторов, их концентраций, pH и природы буферной смеси, а также температуры и продолжительности проведения реакции на активность исследуемой ферментативной системы в бесклеточном экстракте 4-суточной культуры. Зависимость количества образовавшегося агроклавина I от продолжительности реакции представлена в таблице. В качестве стандартной продолжительности проведения реакции было выбрано 3 ч.

Динамика образования агроклавина I во времени при превращении ханоклавина I бесклеточным экстрактом 4-суточной культуры

Продолжительность реакции, ч	Агроклавин I	
	от теоретического	мкг/мл
0	0	0
0,5	22	50
1,0	38	95
3,0	55	130
5,0	61	152
9,0	67	167

Повышение температуры приводило к увеличению выхода агроклавина I: 24° — 42%, 30° — 48% и 37° — 56% агроклавина I. В связи с этим дальнейшие эксперименты проводили при 37°.

Показано, что NADPH и ATP оказывают существенное влияние на ферментативную активность. Наибольшая активность ферментативного комплекса проявляется при содержании NADPH в реакционной смеси от 1,5 до 3,0 мкМ (рис. 4). При содержании ATP в концентрации 10 мкМ ферментативная система более чем в 1,5 раза активна в фосфатном буфере по сравнению с трисацетатным. Оптимум pH находится в области pH 8,0 (рис. 5).

Физиологические особенности гриба *P. sizovae* F-209 и образование им алкалоидов были описаны ранее [5]. Культура синтезирует алкалоиды эрголинового ряда, преимущественно агроклавин I и эпоксиагроклавин I, а при варьировании условий культивирования возможно образование в минорных количествах агроклавина, ханоклавина I и ханоклавина III. На рис. 1 представлена структура этих алкалоидов.

Для биосинтеза эргоалкалоидов у *Claviceps* установлен следующий путь: триптофан→ДМАТ→ханоклавин I→элимоклавин→амиды лизергиновой кислоты [8]. Рядом авторов показано, что бесклеточные экст-

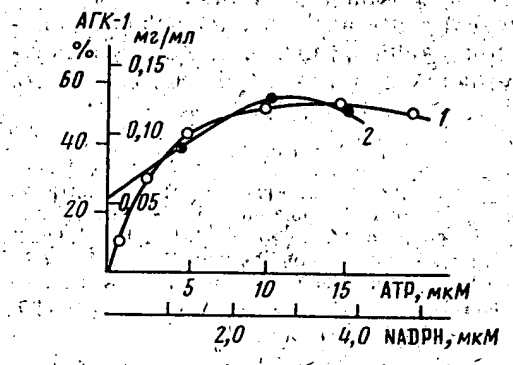

Рис. 4

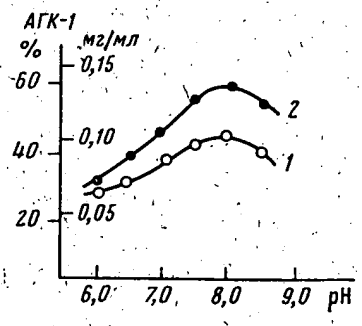

Рис. 5

Рис. 4. Влияние NADPH и ATP на активность ханоклавинциклазы. *1* — NADPH, *2* — ATP

Рис. 5. Влияние природы буфера и pH на активность ханоклавинциклазы. *1* — трисацетатный буфер, *2* — фосфатный буфер

ракты различных штаммов *Claviceps* катализируют превращение ханоклавина I в агроклавин [9] или в элимоклавин без промежуточного образования агроклавина [10]. На основании полученных нами данных об образовании в бесклеточном экстракте мицелия *P. sizovae* ферментативной системы, осуществляющей превращение ханоклавина I, можно предположить, что схема биосинтеза эргоалкалоидов у *P. sizovae* в целом аналогична таковой у *Claviceps*. Однако стереохимия некоторых алкалоидов, образующихся в результате биосинтеза у *P. sizovae* (агроклавин I, ханоклавин III, эпоксиагроклавин I), отличается от таковых у *Claviceps* (рис. 1). Образование в качестве основного продукта реакции при использовании в качестве исходного субстрата ханоклавина I агроклавина I, а не агроклавина, свидетельствует о наличии промежуточного продукта между образованием агроклавина I из ханоклавина I. Обнаружение ханоклавина III в качестве минорного компонента при превращении ханоклавина I бесклеточными экстрактами как 3-, так и 4-суточной культуры *P. sizovae* (рис. 2, 3) подтверждает предположение, что превращение ханоклавина I в агроклавин I у этой культуры может происходить через промежуточное образование ханоклавина III. Исходя из представленных данных можно предложить следующую схему биосинтеза клавиновых алкалоидов *P. sizovae*: агроклавин←ханоклавин I→ханоклавин III→агроклавин I→эпоксиагроклавин I.

Выражаем благодарность И. Г. Веприцкой и Т. А. Решетиловой за предоставление субстрата — ханоклавина I, свидетелей и подбор условий экстракции алкалоидов.

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10.05.1988

Институт биохимии и физиологии микроорганизмов АН СССР, Пущино

N. Yu. MARKELOVA, A. G. KOZLOVSKY

ENZYMATIC TRANSFORMATION OF CHANOCLAVINE-I BY THE *PENICILLIUM SIZOVAE* F-209 CELL-FREE EXTRACT

Institute of Biochemistry and Physiology of Microorganisms, USSR Academy of Sciences, Pushchino

The activity of an enzyme complex transforming chanoclavine-I was observed in cell-free extracts of the fungus *Penicillium sizovae* F-209. The optimal conditions for the enzyme system functioning were chosen: concentration of cofactors, pH and temperature of the reaction mixture, when about 50% of chanoclavine-I is transformed by the mycelial cell-free extract. The main products of the transformation are agroclavine-I and epoxyagroclavine-I.

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TI Ergot alkaloid biosynthesis by isolates of Balansia epichloe and B. henningsiana
AU Bacon, Charles W.; Porter, James K.; Robbins, Joe D.
CS Richard B. Russell Res. Cent., USDA, Athens, GA, 30613, USA
SO Can. J. Bot. (1981), 59(12), 2534-8

TI Conformations of the ergot alkaloids chanoclavine-1, aurantioclavine, and N-acetylaurantioclavine [Claviceps purpurea, Fungi].
AU Sakharovskii, V.G.; Aripovskii, A.V.; Baru, M.B.; Kozlovskii, A.G.
AV DNAL (QD241.K453)
SO Chemistry of natural compounds., Sept/Oct 1983 (pub. 1984) Vol. 19, No. 5. p. 626-627

TI Peptide-type ergot alkaloids produced by Hypomyces aurantius
AU Yamatodani, Saburo; Yamamoto, Isao
CS Kobe Women's Junior Coll., Kobe, 650, Japan
SO Nippon Nogei Kagaku Kaishi (1983), 57(5), 453-6

Biosynthesis of ergot alkaloids. Mechanism of the conversion of chanoclavine-I into tetracyclic ergolines
AU Floss, Heinz G.; Tcheng-Lin, Marie; Chang, Ching-Jer; Naidoo, Bala; Blair, Garre E.; Abou-Chaar, Charles I.; Cassady, John M.
CS Dep. Med. Chem., Purdue Univ., West Lafayette, Indiana, USA
SO J. Amer. Chem. Soc. (1974), 96(6), 1898-909

TI Ergot alkaloid identification in clavicipitaceae systemic fungi of pasture grasses
AU Porter, James K.; Bacon, Charles W.; Robbins, Joe D.; Betowski, Don
CS Richard B. Russell Agric. Res. Cent., United States Dep. Agric., Athens, GA, USA
SO J. Agric. Food Chem. (1981), 29(3), 653-7

TI ALKALOIDS FROM THE FUNGUS CLAVICEPS-SP IBPM-F-401.
AU KOZLOVSKII A G; ARINBASAROV M U; SOLOV'EVA T F; ADANIN V M; GRIGOROV I; ANGELOV T I; SLOKOSKA L S; ANGELOVA M B
CS INST. BIOCHEM. PHYSIOL. MICROORG., ACAD. SCI. USSR, PUSHCHINO, USSR.
SO PRIKL BIOKHIM MIKROBIOL, (1980) 16 (4), 569-577.

90:199927
TI Ergosine, ergosinine, and chanoclavine I from Epichloe typhina
AU Porter, James K.; Bacon, Charles W.; Robbins, Joe D.
CS Richard B. Russell Agric. Res. Cent., Sci. Educ. Adm., Athens, Ga., USA
SO J. Agric. Food Chem. (1979), 27(3), 595-8

I LABORATORY PRODUCTION OF ERGOT ALKALOIDS BY SPECIES OF BALANSIA.
AU BACON C W; PORTER J K; ROBBINS J D
CS US FIELD CROPS LAB., US SCI. EDUC. ADM., R. B. RUSSELL AGRIC. RES. CENT., ATHENS, GA. 30604, USA.

Biosynthesis of Ergot Alkaloids. Studies on the Mechanism of the Conversion of Chanoclavine-I into Tetracyclic Ergolines

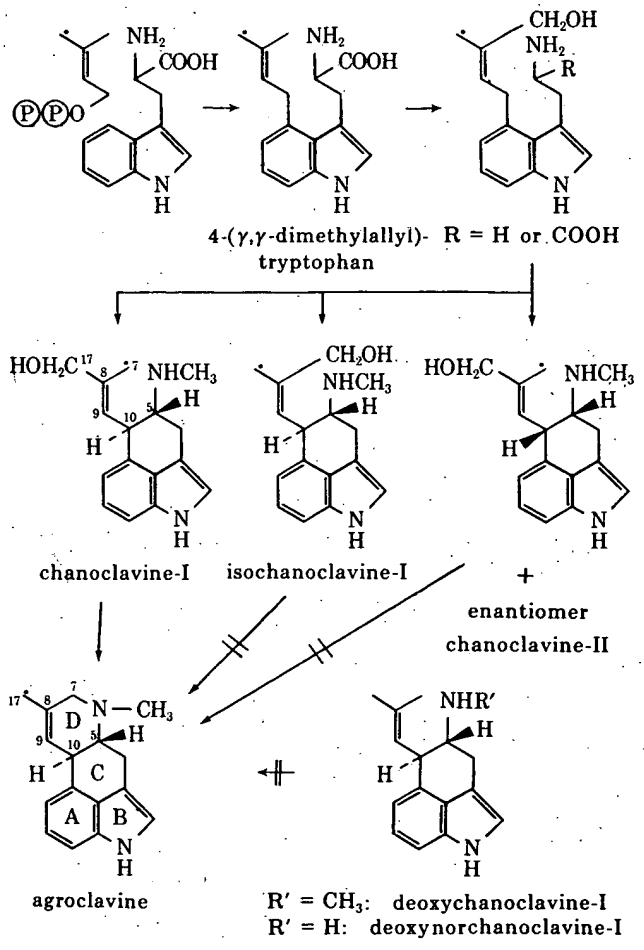
Heinz G. Floss,* Marie Tcheng-Lin, Ching-jer Chang, Bala Naidoo, Garre E. Blair, Charles I. Abou-Chaar and John M. Cassady

Contribution from the Department of Medicinal Chemistry and Pharmacognosy, Purdue University, West Lafayette, Indiana 47907. *Received August 7, 1973*

Abstract: As indicated by double labeling experiments with stable isotopes, the cyclization of chanoclavine-I to tetracyclic ergolines involves an intermolecular transfer of the hydrogen at C-9 into the same position of a new molecule. Also in this process, one of the methylene hydrogens at C-17 of the precursor is exchanged, the newly introduced hydrogen occupying the *pro-R* position at C-7. This and the fact that chanoclavine-I-aldehyde is efficiently and specifically converted into elymoclavine by the ergot fungus suggest that the cyclization involves aldehyde intermediates. Experiments with (3*R*,4*R*)-mevalonic-2-^{14}*C*-4-*t* acid indicate that an isotope effect in the further metabolism of chanoclavine-I leads to an enrichment of tritium at C-9 of the unreacted chanoclavine-I. A mechanism involving double bond isomerization at the aldehyde stage is proposed which accounts for all the observations.

The biosynthesis of the tetracyclic ergoline ring system of ergot alkaloids from tryptophan, mevalonic acid, and the methyl group of methionine has been found to involve an unexpectedly complex sequence of reactions which is still not very well understood.[1] The pathway as we see it now (Scheme I) involves two cis–trans isomerizations at the allylic double bond, one occurring in the reaction sequence leading to chanoclavine-I and the other in the conversion of chanoclavine-I into the tetracyclic ergoline, agroclavine. It is attractive to speculate that these two isomerizations are somehow tied to the corresponding ring closure reactions leading to the formation of the 5,10 and the 6,7 single bonds, respectively. An attempt has been made to establish such a relationship for the first isomerization and the formation of ring C but without producing the expected results.[2,3] Various pieces of information are available, which relate to the mechanism of the second isomerization and the formation of ring D. It is known that the conversion of chanoclavine-I to agroclavine and elymoclavine proceeds with complete retention of the hydrogen at C-10.[4,5] The retention of the hydrogen at C-9, on the other hand, is not complete. Tritium retentions of 70% were observed for the incorporation of both (3*R*,4*R*)-mevalonic-2-^{14}*C*-4-*t* acid and chanoclavine-I-7-^{14}*C*-9-*t* into elymoclavine.[5] The chanoclavine-I reisolated from the latter experiment had the same T/^{14}C ratio as the starting material. The same mevalonic acid was converted into chanoclavine-I with 100% tritium retention using a different ergot strain which produces chanoclavine-I as the terminal alkaloid.[5] In the present paper we would like to present results which give further insight into the mechanism of the transformation of chanoclavine-I into tetracyclic ergolines. Some of these results have been communicated in preliminary form.[6]

Scheme I



Results

In a previous publication[5] we discussed, as one explanation for the above mentioned tritium retention

(1) For reviews see (a) R. Voigt, *Pharmazie*, 23, 285, 353, 419 (1968); (b) E. Ramstad, *Lloydia*, 31, 327 (1968); H. G. Floss, *Abh. Deut. Akad. Wiss. Berlin, Kl. Chem., Geol., Biol.*, 395 (1972).

(2) M. Seiler, W. Acklin, and D. Arigoni, *Chem. Commun.*, 1394 (1970); *Chimia*, 24, 449 (1970).

(3) C. I. Abou-Chaar, H. F. Guenther, M. F. Manuel, J. E. Robbers, and H. G. Floss, *Lloydia*, 35, 272 (1972).

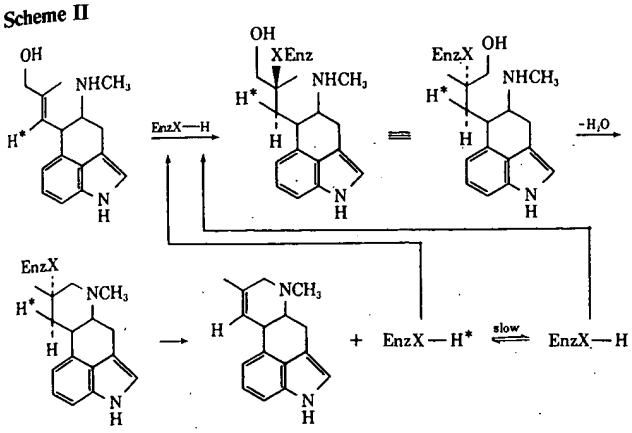
(4) S. Agurell, *Acta Pharm. Suecica*, 3, 71 (1966).

(5) H. G. Floss, U. Hornemann, N. Schilling, K. Kelley, D. Gröger, and D. Erge, *J. Amer. Chem. Soc.*, 90, 6500 (1968).

(6) H. G. Floss, D. Gröger, and D. Erge, paper presented at the 5th International Symposium on the Chemistry of Natural Products, London, July 1968, Abstracts, p 72; B. Naidoo, J. M. Cassady, G. E. Blair, and H. G. Floss, *Chem. Commun.*, 471 (1970).

data, the possibility of a mechanism involving an intermolecular hydrogen transfer. Such a mechanism can, for example, be visualized as shown in Scheme II.

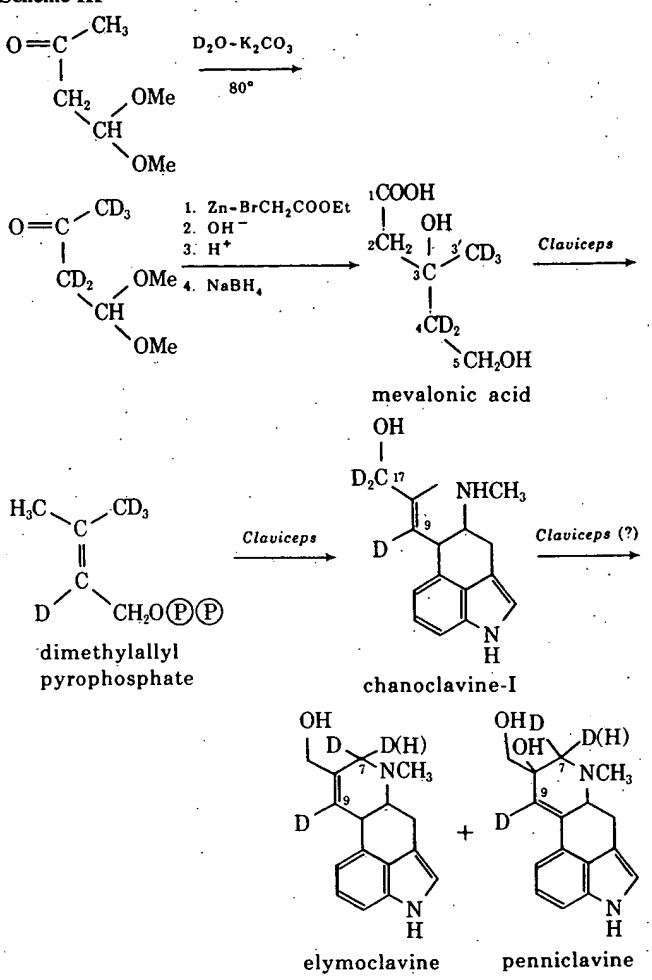
Scheme II



By addition of some XH group of the enzyme across the double bond of chanoclavine-I, a second hydrogen would be introduced at C-9 and, with the rotation around the 8,9 bond required to bring the hydroxymethyl group close to the nitrogen, the original hydrogen from this position would be transferred to the enzyme in the subsequent elimination step. If this hydrogen on the enzyme were to undergo only slow exchange with solvent protons, it could be introduced into a new molecule of bound substrate, where it would now occupy the enantiotopic position and would thus end up in the product molecule upon completion of the second turnover of the enzyme. To test for such an intermolecular transfer of the hydrogen at C-9 of chanoclavine-I we carried out experiments with mevalonic acid intramolecularly labeled with deuterium at C-4 (indicator label) and C-3′ (reference label). This material was prepared as shown in Scheme III. Base-catalyzed equilibration of 1,1-dimethoxybutan-3-one with D_2O gave, after four exchanges, the pentadeuterated compound containing 100% deuterium in the methylene group and over 90% deuterium in the C-methyl group, as determined by pmr. This material was then used in a Reformatsky reaction to give the acetal of mevaldic acid ethyl ester, which was converted to mevalonic acid essentially as described by Eggerer and Lynen.[7] As shown by mass spectral analysis, the latter consisted of 80% pentadeuterated, 11% tetradeuterated, 7% trideuterated molecules, and less than 1% each of molecules containing 2, 1, and 0 atoms of deuterium. The rest of Scheme III indicates the presumed metabolic fate of these labeled hydrogens in the ergot fungus. One atom of deuterium from C-4 would be lost in the transformation of mevalonic acid to dimethylallyl pyrophosphate and another one from the methyl group in the further transformation of the latter into chanoclavine-I. This alkaloid, then, should contain the majority of its deuterium in molecules carrying 3 atoms of deuterium. In addition there would be varying amounts of unlabeled molecules, depending on the ratio of added to endogenously synthesized mevalonic acid, and, because of the incomplete deuteration of the precursor, a small percentage of di- and monodeuterated species. If the conversion

(7) H. Eggerer, F. Lynen, E. Rauenbusch, and J. Kessel, *Justus Liebigs Ann. Chem.*, 608, 71 (1957).

Scheme III



of chanoclavine-I into the tetracyclic alkaloids is straightforward, then elymoclavine and its immediate conversion product penniclavine should have essentially the same deuterium distribution, except for a slight decrease in deuterium content due to exchange or an isotope effect. If on the other hand this conversion involves an intermolecular hydrogen transfer as outlined above, then a deuterium atom removed from C-9 could be introduced either into a molecule already containing deuterium, in which case there would be no net change, or into an unlabeled molecule, in which case one D_3 molecule and one D_0 molecule would give rise to one D_2 and one D_1 molecule. The operation of an intermolecular hydrogen transfer mechanism would thus increase the amount of D_2 and D_1 species in the tetracyclic alkaloids at the expense of the D_3 and the D_0 species. This increase would depend on the ratio of labeled to unlabeled species in chanoclavine-I, and the expected deuterium distribution of the tetracyclic alkaloids can be estimated from the observed deuterium distribution of chanoclavine-I and the degree of tritium retention. To determine the latter individually in each experiment, the deuterated mevalonic acid was mixed with (*3R,4R*)-mevalonic-*2*-^{14}*C*-*4*-*t*[8] acid (final specific activity 1.44 × 10^7 dpm ^{14}C/mmol, T/^{14}C = 2.48). This precursor was then fed to cultures of *Claviceps* strain SD 58 under several different con-

(8) "(3*R*,4*R*)-mevalonic-*2*-^{14}*C*-*4*-*t* acid" always refers to the 1:1 mixture of the 3*R*,4*R* and 3*S*,4*S* isomers, of which only the 3*R*,4*R* isomer is biologically active.[5] The radioactivities given are always those of the mixture.

Table I. Conditions of Feeding Experiments with Intramolecularly Double Labeled Deuteriomevalonic Acid in *Claviceps* strain SD 58 (T/^{14}C of precursor, 2.48)

Expt no.	Feeding conditions	Alkaloid formed	Radioactivity of product (T/^{14}C = T retn)[a]
1	0.2 mmol mevalonate-*2*-^{14}*C*-4*R*-4-*t*-3′,4-d_5 to one 100-ml NL 406 medium replacement culture	4.7 mg	Chanoclavine-I: 3.33 = 134% Elymoclavine: 1.35 = 54% Penniclavine: 1.28 = 52%
2	0.5 mmol mevalonate-*2*-^{14}*C*-4*R*-4-*t*-3′,4-d_5 to two 25-ml NL 406 medium replacement cultures	7.6 mg	Chanoclavine-I: 3.37 = 136% Elymoclavine: 2.04 = 82% Penniclavine: 1.83 = 74%
3	0.05 mmol mevalonate-*2*-^{14}*C*-4*R*-4-*t*-3′,4-d_5 + 0.02 mmol L-tryptophan to two 25-ml phosphate buffer replacement cultures	692 μg	Chanoclavine-I: 3.1 = 127% Elymoclavine: 1.0 = 40%
4	0.025 mmol mevalonate-*2*-^{14}*C*-4*R*-4-*t*-3′,4-d_5 + 0.025 mmol mevalonate + 0.02 mmol L-tryptophan as in expt 3	719 μg	Chanoclavine-I: 3.5 = 141% Elymoclavine: 0.96 = 39%

[a] T retn (tritium retention) = {[T/^{14}C of product]/[T/^{14}C of precursor]}100%.

Table II. Deuterium Distribution in Clavine Alkaloids Biosynthesized from Mevalonic-3′,4-d_5 Acid by *Claviceps* strain SD 58

		Estimated distribution in tetracyclic clavines for—					
				Exchange at C-9 and loss of 1H from C-17	Intermolecular transfer at C-9 and loss of 1H from C-17	Found in—	
%[a]	Found in chanoclavine-I	Exchange at C-9	Intermolecular transfer at C-9			Elymoclavine	Penniclavine
				Experiment 1			
D_3	28.4	15	6	0	0	0.7	0.3
D_2	12.4	20	25	18	6–8	9.1	6.1
D_1	1.8	7	23	21	25–42	28.7	28.9
D_0	57.4	58	46	61	51–69	61.5	64.7
				Experiment 2			
D_3	46.0	36	24	0	0	2.5	6.9
D_2	19.0	25	32	43	24–29	36.3	28.8
D_1	2.9	6	27	22	32–50	26.5	30.8
D_0	32.1	33	17	35	21–44	34.7	33.5
				Experiment 3			
D_3	33.6	13	7	0	0	0.5	
D_2	16.5	27	30	17	7–9	11.4	
D_1	5.7	12	24	31	30–46	32.0	
D_0	44.2	48	39	52	45–63	56.1	
				Experiment 4			
D_3	27.9	11	5	0	0	0.4	
D_2	6.0	19	24	12	5–6	3.2	
D_1	14.7	9	17	26	24–38	20.7	
D_0	51.4	61	54	62	56–71	75.7	

[a] The figures given are the percentages of the molecules containing 3, 2, 1 and 0 atoms of deuterium, respectively.

Table III. Incorporation of ^{14}C- and Tritium-Labeled Precursors into Elymoclavine by *Claviceps* Strain SD 58

Expt no.	Precursor fed	Radioactivity fed	Alkaloid formed, mg	Radioactivity of elymoclavine	Incorporation, % T retn, %
5	18.6 mg chanoclavine-I-^{14}*C-17-t*	1.19×10^6 dpm ^{14}C, T/^{14}C = 5.15	141	2.10×10^5 dpm ^{14}C, T/^{14}C = 2.72	17.6 53
8	(3*R*,4*R*)-Mevalonolactone-*2*-^{14}*C-4-t*	4.4×10^7 dpm ^{14}C, T/^{14}C = 5.55	128	6.15×10^6 dpm ^{14}C, T/^{14}C = 3.16	14 57
14	0.25 mg chanoclavine-I-aldehyde-*17-t*	1.01×10^7 dpm	142	4.05×10^6 dpm	40.1
15	0.06 mg chanoclavine-I-*17-t*	4.88×10^6 dpm	141	4.83×10^5 dpm	9.9
16	0.12 mg chanoclavine-I-aldehyde-*17-t*	5.0×10^6 dpm	127	1.17×10^6 dpm	23.3
17	0.12 mg chanoclavine-I-*17-t*	1.0×10^7 dpm	127	5.74×10^5 dpm	5.7
18	5.2 mg chanoclavine-I-aldehyde-^{14}*C-17-t*	3.60×10^5 dpm ^{14}C, T/^{14}C = 1.78	71	1.24×10^5 dpm ^{14}C, T/^{14}C = 1.61	34.4 91

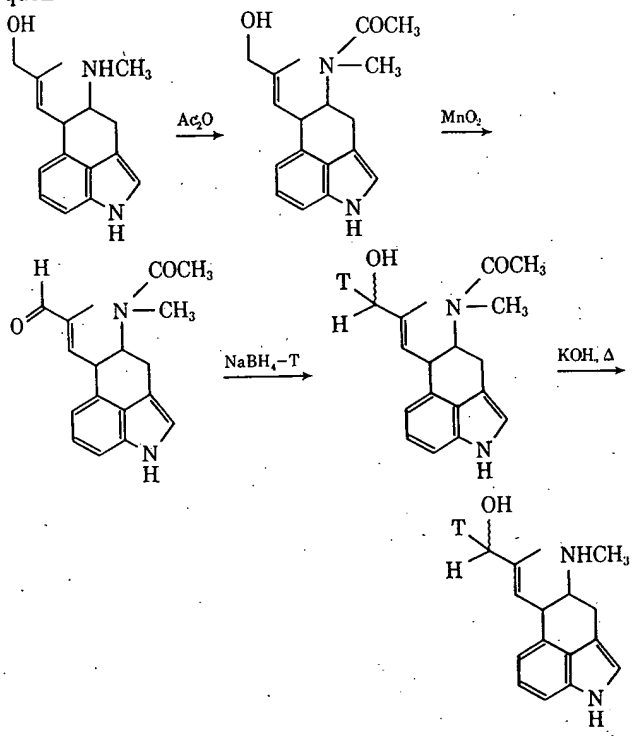
ditions and the alkaloids chanoclavine-I, elymoclavine, and, in some experiments, penniclavine were isolated, purified chromatographically, and analyzed for their T/^{14}C ratios. The data are summarized in Table I. Aliquots of these alkaloids were then subjected to mass spectral analysis to determine from the isotope composition of the molecular ion the number of unlabeled, mono-, di-, and trideuterated molecules. In Table II the observed values are compared with the expected figures or the expected range of values estimated for various mechanisms. In accordance with expectations, the chanoclavine-I does indeed carry most of its

Table IV. Degradation of Biosynthetically Labeled Elymoclavines

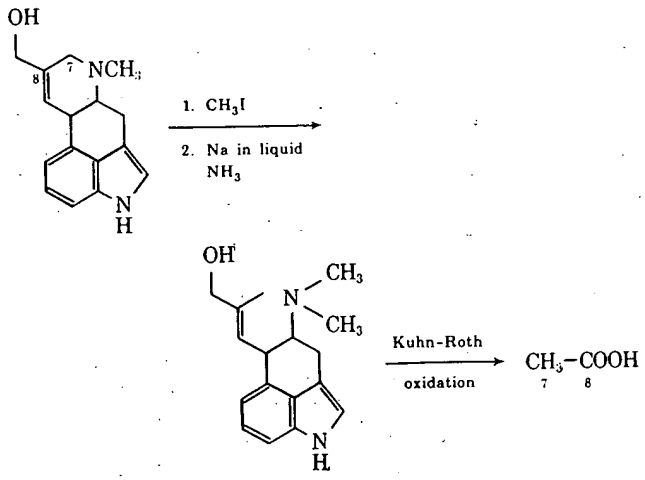
Expt no.	Precursor	Elymoclavine	*N*-Methylsecoelymoclavine	Acetic acid
5	Chanoclavine-I-^{14}C-*17-t*	T/^{14}C = 2.72	2.71×10^5 dpm T/mmol, T/^{14}C = 2.69	2.61×10^5 dpm T/mmol = 93%
8	(3*R*,4*R*)-Mevalonolactone-*2*-^{14}C-*4-t*	2.22×10^6 dpm T/mmol, T/^{14}C = 3.16	2.18×10^6 dpm T/mmol, T/^{14}C = 3.18	3.0×10^3 dpm T/mmol = 0.14%; 4.7×10^3 dpm T/mmol = 0.21%
14	Chanoclavine-I-aldehyde-*17-t*	2.08×10^5 dpm T/mmol	N.d.	2.05×10^5 dpm T/mmol = 98.5%
16	Chanoclavine-I-aldehyde-*17-t*	2.75×10^5 dpm T/mmol	N.d.	2.59×10^5 dpm T/mmol = 94%

deuterium in the form of trideuterated molecules. However, the distribution of deuterium in elymoclavine and penniclavine is quite different. Particularly striking is the virtual absence of trideuterated molecules. This is definitely not in agreement with the expected distribution for a simple exchange of some of the label from C-9 and it also does not fit the values expected for just an intermolecular transfer. However, a much better fit is obtained if the assumption is made that the reaction involves not only an intermolecular transfer of the hydrogen at C-9 but also loss of one of the deuterium reference labels at C-17. Assuming loss of one of the deuterium atoms from C-17 also improves the fit between observed and calculated data for the case of a mere exchange of some label from C-9, although the agreement is not as good as for the intermolecular transfer case.

In order to test the assumption that one of the hydrogens at C-17 is replaced during the transformation of chanoclavine-I into tetracyclic ergolines, we prepared chanoclavine-I-*17-t* by the following reaction sequence.[9,10]



The last step, the alkaline hydrolysis of *N*-acetylchanoclavine-I, is very inefficient[10] and in later work we therefore used direct reduction of chanoclavine-I-aldehyde for the preparation of chanoclavine-I-*17-t*. The tritiated material was mixed with chanoclavine-I-^{14}C obtained biosynthetically from tryptophan-$^{14}C^{11}$ to give a T/^{14}C = 5.15 and then fed to two cultures of *Claviceps* strain SD 58. The resulting elymoclavine (experiment 5, Table III) had a T/^{14}C ratio of 2.72 (tritium retention 53%) confirming the loss of one of the two labeled hydrogens from C-17 of chanoclavine-I. The elymoclavine from this experiment was diluted with carrier material and degraded by the following reaction sequence[5,12] to locate the tritium in the molecule.



The result of this degradation (Table IV) shows that essentially all the tritium is confined to the 7 position as expected. Thus, the experiment with chanoclavine-I-^{14}C-*17-t* confirms the correctness of the assumption that one of the hydrogens at C-17 is replaced during the conversion into tetracyclic ergolines.

While the data from the experiments with mevalonate-*3',4-d*$_5$, in light of the above, favor a process involving an intermolecular hydrogen transfer, the predicted deuterium distributions for exchange at C-9 and loss of one deuterium from C-17 are not sufficiently different to exclude this possibility. Furthermore, the evidence for an intermolecular hydrogen transfer in this type of experiment is based on the separation of two isotopic labels which in the precursor are present in the same molecule. A much stronger case could be made on the basis of an experiment of the opposite type, in which two isotopic labels which are present in separate molecules in the precursor end up in the same molecule in the product. To carry out such an experiment we synthesized mevalonic-*4-d*$_2$ acid from acetic-*d*$_4$ acid *via* methyl bromoacetate and mevaldic acid essentially as described by Cornforth, *et al.*,[13] for the synthesis of

(9) T. Fehr, Ph.D. Dissertation, ETH Zürich, 1967.
(10) A. Hofmann, R. Brunner, H. Kobel, and A. Brack, *Helv. Chim. Acta*, 40, 1358 (1957).
(11) D. Gröger, D. Erge, and H. G. Floss, *Z. Naturforschg. B*, 21, 827 (1966).
(12) S. Bhattacharji, A. J. Birch, A. Brack, A. Hofmann, H. Kobel, D. C. C. Smith, H. Smith, and J. Winter, *J. Chem. Soc.*, 421 (1962).
(13) J. W. Cornforth, R. H. Cornforth, A. Pelter, M. E. Horning, and G. Popjak, *Tetrahedron*, 5, 311 (1959).

mevalonic-4-^{14}C acid. This material (99% D_2) was mixed in a ratio of 1:1 with mevalonic acid-2-^{13}C which had been synthesized from sodium acetate-2-^{13}C (86.8 atom % excess ^{13}C) *via* ethyl acetate and its condensation with 1,1-dimethoxybutan-3-one according to Pichat, *et al.*,[14] to give the ethyl ester of mevaldic acid dimethyl acetal, which was then converted to mevalonic acid by a modification of the method of Eggerer, *et al.*[7] This mixture was fed in each of two separate experiments to two 20-ml medium NL 406 replacement cultures of *Claviceps* strain SD 58. Chanoclavine-I and elymoclavine were isolated and purified as before and analyzed by mass spectrometry. Since the quantitative determination of the isotope composition of elymoclavine is somewhat complicated by the presence of a strong M-1 peak in the mass spectrum, a portion of each of the two elymoclavine samples was treated with horseradish peroxidase and hydrogen peroxide as described by Ramstad and coworkers[15] and the resulting penniclavine was purified and also subjected to mass spectral analysis. The absence of an M − 1 peak in $\Delta^{9,10}$-clavines like penniclavine considerably simplifies the calculation of the isotope composition. The percentage of tritium retention from (3*R*,4*R*)-mevalonic-2-^{14}C-4-*t* acid was in this case determined in a separate identical experiment and found to be 65%. The results of these two experiments are shown in Table V, which lists the isotope distribution in excess of the natural abundance. As expected the chanoclavine-I from both experiments shows isotope excess only in the M + 1 peak; *i.e.*, it only contains molecules labeled with deuterium or with ^{13}C in addition to unlabeled ones but none containing both ^{13}C and deuterium. In contrast, the elymoclavine from these two experiments as well as the penniclavine derived from it showed appreciable enrichment in the M + 2 peak indicating the presence of molecules containing both ^{13}C and deuterium. High resolution peak matching showed that the exact mass of the major peak at *m/e* 256 in the elymoclavine spectrum corresponded to the composition $C_{15}{}^{13}CH_{17}DN_2O$.[16] The percentage of doubly labeled molecules in both experiments is well within the calculated range. The lower limit of the predicted range is obtained by making the assumption that the ratio of labeled to unlabeled molecules in chanoclavine-I measured at the end of the experiment represents that throughout the culture period. The upper limit follows from the opposite extreme, assuming that all the labeled alkaloid was synthesized at one time and all the unlabeled at a different time. As would be expected the observed values lie between these extremes. These data thus strongly support the idea of an intermolecular transfer of the hydrogen at C-9 during the conversion of chanoclavine-I into tetracyclic ergolines.

Table V. Isotope Distribution in Clavine Alkaloids Biosynthesized from a Mixture of Mevalonic-2-^{13}C and -4-d_2 Acid (43 Atom % ^{13}C Excess and 50% D_2 in Mixture)[a]

	Percentage of			
	Unlabeled	Single labeled (D or ^{13}C)	Double labeled (D + ^{13}C)	Triple labeled
	Experiment 6			
Chanoclavine-I	58	42	0	0
Elymoclavine	67.1	26.1	6.8	0
Penniclavine	67.0	25.4	7.1	0.5
Predicted for elymoclavine and penniclavine			2.9–14	0
	Experiment 7			
Chanoclavine-I	50.2	49.8	0	0
Elymoclavine	61.7	30.0	8.2	0.1
Penniclavine	59.0	31.8	8.8	0.5
Predicted for elymoclavine and penniclavine			4.0–14	0

[a] Amount of precursor fed: experiment 6, 190 μmol; experiment 7, 200 μmol. Alkaloid formed 19.3 and 30.1 μmol.

If the hydrogen at C-9 undergoes an intermolecular transfer, the question arises whether this transfer occurs back into the same position or whether the label from C-9 of chanoclavine-I appears at a different position in the product. Extensive studies by various groups[1b,17] have established the origin of almost all the hydrogens in the tetracyclic ergolines to be the corresponding hydrogens of the respective precursor. The only two positions in the molecule for which such proof is missing, and to which a hydrogen from C-9 could therefore potentially have migrated, are position 2 at the indole ring and position 7. Transfer of hydrogen from C-9 to C-2 appears as an extremely unlikely prospect, but transfer to C-7 has to be seriously considered in view of the finding that one of the hydrogens from C-17 of chanoclavine-I is replaced in the process. To examine this possibility, we prepared elymoclavine from (3*R*,4*R*)-mevalonate-2-^{14}C-4-*t* (experiment 8, Table III) and degraded it to determine the amount of tritium present at C-7. The results (experiment 8, Table IV) clearly show that C-7 of elymoclavine carries no tritium, thereby excluding the possibility of a hydrogen transfer from C-9 to C-7. By way of exclusion this suggests that the hydrogen from C-9 of chanoclavine-I is transferred to the same position in the tetracyclic product. This tentative conclusion was confirmed by feeding mevalonate-4-d_2 (0.57 mmol of 99% D_2) to 40-ml replacement cultures of *Claviceps* strain SD 58 and analyzing the resulting elymoclavine by high resolution proton nmr. Mass spectrometry of the isolated elymoclavine indicated the presence of 51.5% monodeuterated molecules (D_2 < 1%), and the decrease in the intensity of the pmr signal for H-9 (6.80 ppm)[18] showed that essentially all the deuterium (47%) was located in this position.

Some further, although circumstantial, evidence for a partial intermolecular hydrogen transfer is obtained from an inspection of the data in Table I. The tritium retention values for the tetracyclic clavines vary from

(14) L. Pichat, B. Blagoev, and J. C. Hardouin, *Bull. Soc. Chim. Fr.*, 4489 (1968).

(15) W.-n. Chan-Lin, E. Ramstad, and E. H. Taylor, *Lloydia*, 30, 202 (1967); W.-n. Chan-Lin, Ph.D. Thesis, Purdue University, 1967.

(16) There is one ambiguity. The composition of this ion could also be $C_{15}{}^{13}CH_{19}N_2O$, because the mass of two H is almost identical with that of one D and these two possibilities cannot be distinguished with the normal present day high resolution mass spectrometers. However, unlabeled elymoclavine produces almost exactly the theoretical distribution of the isotope satellites of the molecular ion and there is thus no indication for a proton addition process in the mass spectrometer.

(17) B. J. Wilson, Ph.D. Dissertation, Purdue University, 1970.

(18) A complete analysis and reinterpretation of the pmr spectra of agroclavine and elymoclavine has been done (E. C. Kornfeld, N. J. Bach, H. E. Boaz, C.-j. Chang, H. G. Floss, E. W. Hagaman, and E. Wenkert *J. Org. Chem.*, in press).

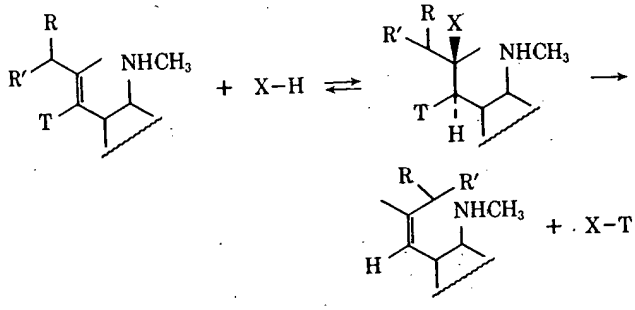
experiment to experiment in a way which seems to correlate with the amount of alkaloid formed. At these very low rates of alkaloid synthesis the tritium retention seems to decrease with decreasing rate of alkaloid formation. This could be very plausibly explained by the working hypothesis illustrated in Scheme II, because at low rates of alkaloid synthesis the enzyme would be idle for longer periods of time between turnovers and labeled hydrogens on the enzyme would have more time to undergo exchange with solvent protons. However, it is somewhat difficult to judge how valid this observation and its interpretation is, because the tritium retention values for the tetracyclic ergolines do show some general fluctuation throughout these experiments. More significant is the finding that chanoclavine-I in all four experiments has a higher T/^{14}C ratio than the precursor. A systematic experiment with (*3R,4R*)-mevalonic-*2*-^{14}*C-4-t* acid (Table VI) indicates that

Table VI. Incorporation of (*3R,4R*)-Mevalonic-*2*-^{14}*C-4-t* Acid into Clavine Alkaloids by *Claviceps* Strain SD 58 (Experiment 9)[a]

Compd	T/^{14}C	T retn, %
Precursor	5.00	
Agroclavine	3.14	63
Elymoclavine	2.61	52
Chanoclavine-I	7.29	146
(−)-Chanoclavine-II	5.25	105
Isochanoclavine-I	4.97	99

[a] Total alkaloid formed 126.5 mg, ^{14}C incorporation 13.75%.

chanoclavine-I is the only clavine showing this behavior. The tetracyclic clavines show the usual decrease in the T/^{14}C ratio and the other two chanoclavine isomers, which are not precursors for the tetracyclic alkaloids, have the same T/^{14}C ratio as the starting mevalonate.[19] This result implies that an isotope effect operates in the further metabolism of chanoclavine-I which leads to enrichment of tritium in the unreacted chanoclavine-I. This requires the addition of another hydrogen at C-9, followed by competition between these two hydrogens in a subsequent elimination step and complete reversibility of the entire reaction sequence from chanoclavine-I to this elimination step. The following system would satisfy these requirements.



Elimination of X–H would be favored over elimination of X–T, increasing the relative rate of back reaction for those molecules of the addition product which were generated directly from tritiated precursor. If this is true, addition of X–D instead of X–H should decrease the enrichment of tritium in unreacted chanoclavine-I,

(19) The same experiment was carried out by Arigoni and coworkers with a different ergot strain and produced almost identical results. We thank Professor Arigoni for informing us of his results.

because the competition would now be between tritium and deuterium rather than between tritium and hydrogen. An indication that this might be the case can already be seen in experiments 3 and 4 (Table I). These two experiments are identical except for the fact that the mevalonate added in one case was all (except for the traces of radioactive material) mevalonate-*3′,4-d*$_5$ whereas in the other it was a mixture of equal parts of pentadeuterated and nondeuterated material. In the latter case chanoclavine-I has a tritium retention of 141%, which in the presence of all deuterated mevalonate is decreased to 127%. Experiments 10–13 (Table VII) with mevalonate-*4-d*$_2$ show the same trend. The

Table VII. Tritium Retention in Clavines from (*3R,4R*) Mevalonate-*2*-^{14}*C-4-t* in the Presence of Deuterated Carrier Mevalonate[a]

Expt no.	Additive	T/^{14}C of mevalonate	Elymoclavine T/^{14}C	Elymoclavine T retn, %	Chanoclavine-I T/^{13}C	Chanoclavine-I T retn, %
10	186 μmol mevalonate-*4-d*$_2$	2.50	1.58	63	2.84	114
11	Same as 10	2.46	1.65	67	2.87	117
12	93 μmol mevalonate-*4-d*$_2$ + 93 μmol unlabeled mevalonate	2.56	1.50	59	3.19	125
13	Same as 12	2.36	1.55	66	3.08	130

[a] In each experiment 4.5 × 10^6 dpm ^{14}C precursor were fed.

tritium retention in chanoclavine-I of 125 and 130% in the presence of equimolar mixtures of deuterated and nondeuterated mevalonate is decreased to 114 and 117% upon adding only deuterated mevalonate. Somewhat surprisingly the tritium retention of elymoclavine shows little change. It will be noted, of course, that these four experiments also again support the idea of an intermolecular transfer of the hydrogen at C-9. Since the tritium and the deuterium as well as the ^{14}C are present strictly in separate molecules, the observed effect can only have come about if deuterium (or tritium) had been transferred into different molecules.

The loss of one of the labeled hydrogens from C-17 of chanoclavine-I during the conversion into tetracyclic ergolines can be explained if one assumes that this hydroxymethyl group goes through the oxidation stage of an aldehyde in the process. To examine this possibility, we prepared chanoclavine-I-aldehyde. Treatment of chanoclavine-I with MnO_2 in acetone at room temperature gave no reaction, but upon refluxing the mixture for 45 min the aldehyde was produced in 50–60% yield. It was purified by preparative layer chromatography and could be converted back into chanoclavine-I by reduction with $NaBH_4$. Its structure was proven[6] on the basis of the spectroscopic data given in the Experimental Section, which are in agreement with literature data of tricyclic and tetracyclic ergolines.[9,20,21] A sample of chanoclavine-I-aldehyde tritiated at C-17 was then prepared by reducing nonlabeled aldehyde

(20) M. Barger, J. A. Weisbach, B. Douglas, and G. O. Dudek, *Chem. Ind. (London)*, 1072 (1965).
(21) D. Stauffacher and H. Tscherter, *Helv. Chim. Acta*, 47, 2186 (1964).

with $NaBH_4$-*t* and reoxidizing the tritiated chanoclavine-*17-t* with MnO_2. This material was fed to two cultures of *Claviceps* strain SD 58 and as a control two parallel cultures received chanoclavine-I-*17-t* (experiments 14 and 15, Table III). The incorporation of tritium from the aldehyde into elymoclavine was about four times higher than that from chanoclavine-I-*17-t*. Even after correction for loss of half of the tritium from chanoclavine-I-*17-t* in the process, the aldehyde is still twice as efficient as chanoclavine-I as a precursor to elymoclavine. The high rate of incorporation leaves little doubt in a very direct precursor relationship of the aldehyde. Degradation of the elymoclavine from experiment 14 (Table IV) confirmed that the tritium was entirely located in the expected position and thus indicated that the incorporation of the aldehyde was specific. A complete repetition of these two experiments (experiments 16 and 17, Table III and IV) produced very similar results. In another experiment it was examined whether the hydrogen at C-17 of chanoclavine-I-aldehyde is completely retained during the conversion into elymoclavine. Within the limits of experimental error this seems to be the case, since chanoclavine-I-aldehyde-^{14}*C-17-t*, prepared by oxidation of chanoclavine-*I*-^{14}*C-17-t* ($T/^{14}C$ = 3.50), is incorporated with little change of the $T/^{14}C$ ratio (experiment 18, Table III).

To further confirm that the loss of one of the methylene hydrogens from the hydroxymethyl group of chanoclavine-I is not merely an unphysiological process which occurs when the alkaloid is added to the cultures, we fed mevalonate-*3'-d*$_3$ (>98% D_3) and determined the number of deuterium atoms in chanoclavine-I and elymoclavine. The precursor was synthesized by reaction of the THP ester of acetic acid-d_4 with allylmagnesium bromide, followed by ozonolysis of the resulting methyldiallylcarbinol and reductive cleavage of the ozonide with $NaBH_4$.[22] The final oxidation of the 3-methylpentane-1,3,5-triol with silver carbonate–Celite[22] did not work in our hands, but oxidation with CrO_3 in acetic acid as described by Goeggel[23] did produce mevalonolactone, albeit in low yield. This material (570 μmol, as sodium salt) was fed to 40 ml replacement cultures and the resulting chanoclavine-I and elymoclavine were analyzed by mass spectrometry (Table VIII). It is evident that while chanoclavine-I consists of predominantly dideuterated molecules, the deuterium in elymoclavine is present almost exclusively in monodeuterated molecules. The presence of about 4% D_3 species in chanoclavine-I and D_2 species in elymoclavine can be accounted for by the known[1] slight (5–10%) scrambling of the label between the carbon atoms derived from C-2 and C-3′ of mevalonic acid. In support of this, the 220-MHz ^{1}H nmr spectrum of the elymoclavine integrates for about 12.5% D in the C-17 protons (4.45 ppm). The majority of the deuterium (37.8%) resides in the equatorial hydrogen at C-7 (3.65 ppm) with little or no deuterium (5.5%) in the axial hydrogen (3.08 ppm).[24] Thus the deuterium occupies the *pro*-7*S* position whereas the newly introduced hydrogen is the *pro*-7*R* hydrogen.

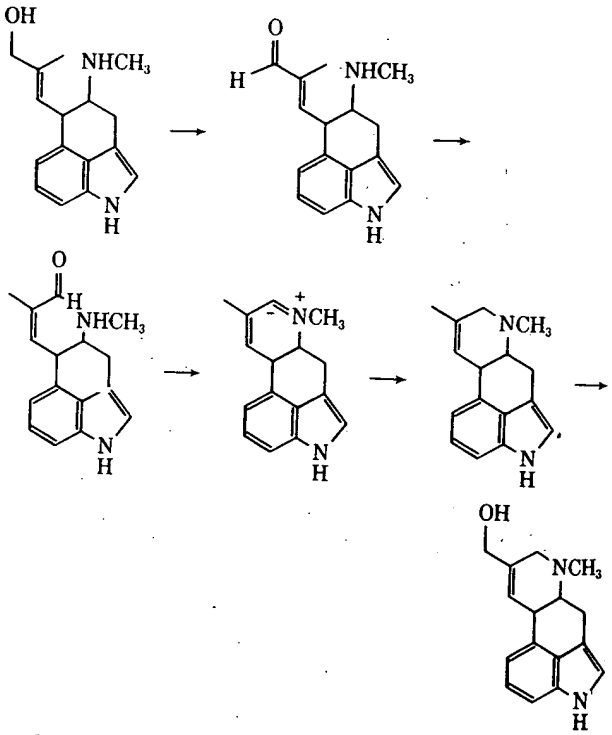
Table VIII. Deuterium Distribution (%) in Chanoclavine-I and Elymoclavine after Feeding Mevalonate-*3'-d*$_3$ to *Claviceps* Strain SD 58

	Labeled species in Chanoclavine-I	Labeled species in Elymoclavine
D_0	21.7	28.2
D_1	11.3	67.9
D_2	63.0	3.9
D_3	4.0	

Discussion

The results of this study clearly indicate that chanoclavine-I-aldehyde can be efficiently and specifically converted into tetracyclic ergolines by the ergot fungus. The fact that it is a more efficient precursor than chanoclavine-I, the finding that the latter is incorporated with loss of half of the tritium from the hydroxymethyl group, and the observation that mevalonate-*3'-d*$_3$ gives rise to chanoclavine-I-d_2 but elymoclavine-d_1 all suggest that the aldehyde is a normal intermediate in the pathway. The conversion of chanoclavine-I into elymoclavine could then be written as shown in Scheme IV.

Scheme IV



The double bond isomerization would take place at the aldehyde stage, leading to isochanoclavine-I-aldehyde which by Schiff base formation and reduction would give rise to agroclavine. Presumably, isochanoclavine-I cannot be dehydrogenated to the aldehyde and is therefore not converted into agroclavine and elymoclavine. Alternatively, as discussed below, the cyclization could lead directly to the carbinolamine. However, there is no definitive evidence that the reaction really proceeds through chanoclavine-I-aldehyde and this pathway therefore still has to be considered as hypothetical.

(22) M. Fetizon, M. Golfier, and J.-M. Louis, *Chem. Commun.*, 1118 (1969).

(23) H. Goeggel, Ph.D. Dissertation No. 3923, ETH Zürich, 1966.

(24) The numerical discrepancy with the mass spectral analysis of elymoclavine could well be the result of an isotope effect. As the fragmentation of elymoclavine is most likely initiated by loss of a hydrogen from C-7, which in this sample carried both H and D, one might expect to find a too high percentage of D_1 species in the molecular ion.

For example, the aldehyde could be reduced to chanoclavine-I and then be converted to tetracyclic ergolines by a different route. A number of trapping experiments, in which labeled aldehyde was fed together with nonlabeled chanoclavine-I and *vice versa* and the unlabeled component reisolated to determine whether it had acquired radioactivity, did indicate some conversion of aldehyde to chanoclavine-I but were inconclusive regarding the formation of the aldehyde from chanoclavine-I, because we were unable to recover any aldehyde from the cultures. If the added aldehyde were indeed incorporated *via* chanoclavine-I, the loss of half of the tritium from C-17 of chanoclavine-I would require a different explanation. A very rapid redox reaction either at the stage of chanoclavine-I or at the tetracyclic stage could account for this finding, provided the tritium on the reduced form of the coenzyme could rapidly equilibrate with a large pool of nonlabeled hydrogens. Such a rapid exchange is unlikely at the chanoclavine-I stage, because in experiments 1–4 (Tables I and II) the chanoclavine-I isolated contains the majority of its deuterium in trideuterated molecules and because the material from mevalonate-3′-d_3 is largely dideuterated (Table VIII). There appears to be no experimental data which would allow an evaluation of the possibility of exchange at the agroclavine or elymoclavine stage. However, this alternative explanation of the experimental observations, although it cannot be excluded, seems to be considerably less likely.

The data on the mechanism of the double bond isomerization seem to clearly support the involvement of an intermolecular hydrogen transfer. While the experiments with mevalonate-*3′,4-*d_5 are little more than indicative, the results of the feeding experiments with a mixture of mevalonate-*2-*^{13}C and *-4-*d_2 can hardly be explained in any other way, and the conclusion is further supported by the effect which mevalonate-*4-*d_2 has on the tritium enrichment at C-9 of unreacted chanoclavine-I. An intermolecular hydrogen transfer requires that the enzyme either binds two molecules of substrate and transfers a hydrogen directly between them or, more likely, that the hydrogen is transferred from one substrate molecule to some acceptor, where it undergoes only little equilibration with other hydrogens while the product dissociates from the enzyme, and then back to a newly bound molecule of substrate. If the hydrogen is transferred as a hydride, the assumption of only limited equilibration with other hydrogens presents no problem. However, even if the hydrogen is transferred as a proton, the assumption of only limited exchange with solvent protons during almost one entire cycle of the enzyme reaction is not unreasonable in view of the recent demonstrations of such very slow proton exchanges, for example, in the aconitase reaction[25] and in the fumarase reaction.[26] Scheme V, which is more or less a modification and extension of the original Scheme II, represents an attempt to accommodate all these various results and considerations in one chemical mechanism. Other schemes can certainly be drawn which also account for all the observations, and we do not mean to imply that the one outlined here is necessarily the best or the only one to describe this process. For example, all the data can also be accommodated by a mechanism involving reversible 1,4-reduction of the α,β-unsaturated carbonyl system of the aldehyde. Modifying Scheme II to account for an aldehyde intermediate obviously presents the problem of having to carry out an addition of X–H across the double bond in an "anti-Michael" direction. However, this problem can be overcome by suitably masking the carbonyl group on the enzyme. In the proposed model the α,β-unsaturated carbonyl portion of the substrate would insert into the crevice of the enzyme in such a way that the groups —Y: and —B:H of the enzyme are situated on one side of the plane of the π system and the groups —X: and —A:H on the other. Following binding through —Y: and —X: and protonation of the oxygen and C-9, partial rotation around the C-8/C-9 axis on the side opposite —B: will bring N-6 in proximity to the aldehyde carbon and the group —A: on the enzyme. The nitrogen atom could thus transfer its proton to —A: and displace —Y: at the aldehyde carbon atom (pathway a). Subsequent removal of —X: and the asterisked hydrogen from C-9 would then release the carbinolamine from the enzyme. —B:H* would then introduce the original hydrogen (H*) from C-9 of this chanoclavine molecule into the next molecule of substrate which binds to the enzyme. As an alternative, the reaction sequence may lead to the release of isochanoclavine-I-aldehyde rather than the carbinolamine from the enzyme as shown in pathway b. One of the attractions of this scheme is that it explains readily the occurrence of an isotope effect leading to tritium enrichment in unreacted chanoclavine-I without any need of resorting to nonstereospecific reactions. All that is required is that every reaction in the scheme and also the conversion of chanoclavine-I to the aldehyde is reversible and that the elimination of a proton from C-9 (at least in the forward reaction) occurs in a rate-limiting step. Both these are entirely plausible assumptions.

Finally, let us consider some of the other mechanisms which have been proposed for the formation of ring D in light of the above data. Clearly, mechanisms which involve isomerization and ring closure merely *via* formation of a carbonium ion at C-17, as both Agurell[4] and we[27] have discussed, are insufficient to account for the new data. Ogunlana, *et al.*,[28] obtained a cell-free system from *Claviceps* which converted chanoclavine-I, but not agroclavine, into elymoclavine in the presence of oxygen, ATP, Mg^{2+}, and NADPH. On the basis of these findings they propose a mechanism which involves (1) phosphorylation of the hydroxyl group, (2) double bond shift from the 8,9 to the 7,8 position, (3) epoxidation of the 7,8 double bond, (4) elimination of phosphate and reaction of the resulting carbonium ion with N-6, (5) opening of the epoxide ring with loss of a proton from either C-9 or from the original C-17 followed by shift of the double bond into the 8,9 position. This mechanism is difficult to reconcile with the above data. To account for the intermolecular hydrogen transfer, steps 2–5 would all have to occur at the same active site of just one enzyme, which is not a very

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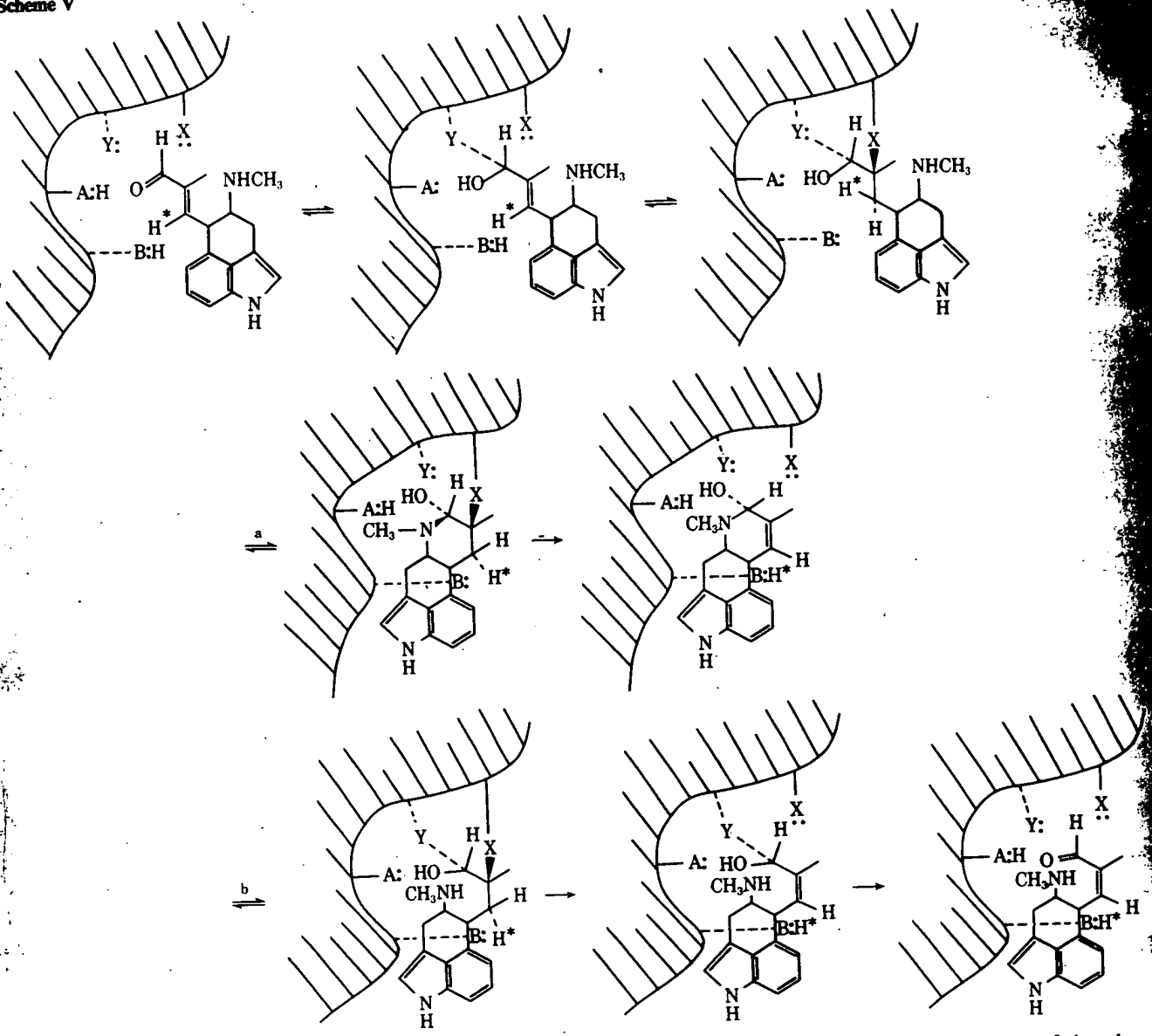
(27) H. G. Floss, U. Hornemann, N. Schilling, D. Gröger, and D. Erge, *Chem. Commun.*, 105 (1967).
(28) E. O. Ogunlana, B. J. Wilson, V. E. Tyler, and E. Ramstad, *Chem. Commun.*, 775 (1970).

Scheme V



likely prospect. To account for the isotope enrichment in unreacted chanoclavine-I, all the steps would have to be completely reversible, which is even less likely.[29-32] Another mode of cyclization of the tricyclic to the tetracyclic ring system involves the transformation of dihydrochanoclavines into dihydroagroclavines in *Claviceps*, which has recently been demonstrated by Voigt and coworkers.[33] The idea of cyclization at the dihydro stage is not incompatible with most of the above results, if one assumes that reduction, ring closure, and dehydrogenation all take place reversibly on the same enzyme; *i.e.*, the dihydro alkaloids would probably not be free intermediates. However, Voigt's group has not observed formation of agroclavine in their experiments[33] and we are therefore inclined to consider the cyclization at the dihydro stage as not to be involved in the transformation of chanoclavine-I to agroclavine. This view is supported by recent experiments of Gröger, Voigt, and their coworkers.[34]

Experimental Section

Feeding Experiments. *Claviceps sp.*, strain SD 58,[35,36] was used throughout this study. The organism was grown in shake culture at 25° in 500-ml Erlenmeyer flasks containing 100 ml of medium NL 406[35,36] for 5 days before the addition of labeled precursors. The normal feeding experiments (experiments 5, 8, 9, 14–18) were carried out and the alkaloids were isolated from them exactly as described previously.[2,35] All alkaloid samples were purified to constant specific radioactivity or constant T/^{14}C ratio either by

(29) Gröger and coworkers[30] have since obtained a cell-free system from *Claviceps* which cyclizes chanoclavine-I under conditions similar to those of Ogunlana, *et al.*,[28] but produces only agroclavine rather than elymoclavine. Their system is not oxygen dependent and does convert chanoclavine-I-aldehyde to agroclavine. We[31] have also obtained a cell-free system from *Claviceps* strain SD 58 which converts chanoclavine-I into tetracyclic ergolines, but our system produces both agroclavine and elymoclavine and, unlike that of Ogunlana, *et al.*, also converts agroclavine into elymoclavine. The difference seems to be primarily in the relative amount of agroclavine hydroxylase[32] present in the system. We therefore see no need to invoke a mechanism for the direct formation of elymoclavine from chanoclavine-I without agroclavine as an intermediate.

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recrystallization (if necessary with carrier) or by repeated chromatography in different systems. For replacement cultures, mycelia were grown in NL 406 to the age of 5–7 days, filtered and washed with water under sterile conditions, and resuspended at twice the ratio of cells to medium in either fresh medium NL 406 (experiments 1, 2, 6, 7, 10–13 and experiments with mevalonate-*4-d*$_2$ and -*3'-d*$_3$) or $^1/_{15}$ *M* phosphate buffer (Soerensen), pH 7.3 (experiments 3 + 4). Labeled precursors were then added immediately and the cultures were incubated with shaking for another 3 days (buffer replacement) or 7–8 days (medium NL 406 replacements). In all the replacement experiments the alkaloids were extracted with 2-propanol–chloroform from the alkalinized culture filtrate and partitioned between 2% succinic acid and methylene chloride. However, instead of the usual column chromatography they were then resolved by preparative tlc on silica gel H with ethanol–chloroform 1:3 as the developing solvent. Except in experiments 3 and 4, the alkaloids were then rechromatographed on silica gel H, chanoclavine-I with *tert*-butyl alcohol–$CHCl_3$ 1:3 in an atmosphere of NH_3, and elymoclavine and penniclavine with ethyl acetate–ethanol–dimethylformamide 85:10:5 as developing solvents. The alkaloids were eluted from the silica gel with 2% succinic acid and the eluate was extracted twice with ether (reagent grade), made alkaline with concentrated NH_4OH, and extracted five times with 4 ml of ether. These operations were carried out in test tubes using Pasteur pipets for the transfers in order to avoid contamination with silicone grease or plasticizer. The combined second ether extract was dried over sodium sulfate and evaporated to dryness. The samples were then taken up in a small amount of methanol and evaporated in melting point capillaries for mass spectral analysis.

The amount of total alkaloid formed was determined colorimetrically with *p*-dimethylaminobenzaldehyde[37,38] and is expressed as milligrams of elmoclavine (mol wt 254).

Labeled Precursors. (3*R*,*S*)-Mevalonic-*2*-^{14}*C* acid, (3*R*,4*R*)-mevalonic-*4-t* acid, and $NaBH_4$-*t* were obtained from Amersham-Searle, Inc. and sodium acetate-*2*-^{13}*C* and acetic-d_4 acid from Prochem Ltd. Chanoclavine-I-^{14}*C* has been biosynthesized from tryptophan-^{14}*C* using *Claviceps paspali* strain Li 342/SE 156[11] and was a gift from Professor Gröger, Halle. Mevalonic-*4*-d_2 acid dibenzylethylenediamine salt (mp 120°) was synthesized by the method of Cornforth, *et al.*,[13] from methyl bromoacetate which in turn had been prepared from acetic-d_4 acid.

Mevalonic-*3',4*-d_5 Acid. 1,1-Dimethoxybutan-3-one (39.6 g) (Chemische Fabrik Hüls, Marl, Germany), 60 ml of D_2O (99.7% D), and 3 g of potassium carbonate were heated to 80° for 8 hr with protection from atmospheric moisture. The mixture was then extracted with ether, the ether evaporated, and the exchange repeated twice. Extensive decomposition took place during the exchange as evidenced by the appearance of a red color and a crystalline product in the reaction mixture. After the third exchange the product was distilled (71–72°, 14 Torr) and combined with that from a second identical run to yield 15.7 g of material which by ^{1}H nmr analysis contained ~100% D in the methylene group and 86.5% D in the *C*-methyl group. This material was subjected to one more exchange with 40 ml of D_2O and 2 g of potassium carbonate at 80° for 15 hr. Extraction and distillation gave 3.95 g of 1,1-dimethoxybutan-3-one-*2,4*-d_5, which according to the mass spectrum contained 78.2% D_5, 13.3% D_4, 6.4% D_3, and less than 1% each of D_2, D_1, and D_0 species. Reformatzki reaction of 3.8 g of this acetal with ethyl bromoacetate according to Cornforth, *et al.*,[13] gave 2.175 g of 3-hydroxy-3-methyl-5,5-dimethoxyvaleric acid ethyl ester (bp 69–73°, 10^{-3} Torr, 34.3% yield), which was converted into mevalonic-*3',4*-d_5 acid dibenzylethylenediamine salt (mp 121–121.5°, 19.4% yield) as described by Eggerer, *et al.*[7] Analysis by mass spectrometry as the lactone indicated the presence of 82.2% D_5, 8.9% D_4, and 8.9% D_3 species and essentially no di-, mono-, or undeuterated species. Averaged with the figures for the 1,1-dimethoxybutan-3-one the mevalonic acid was assumed to consist of about 80% D_5, 11% D_4, 7% D_3, and <1% each of D_2, D_1, and D_0 species.

Mevalonic-*2*-^{13}*C* Acid.[14] Sodium acetate-*2*-^{13}*C* (0.5 g = 6 mmol, 86.8% enrichment) was converted into ethyl acetate-*2*-^{13}*C* by the method of Ropp.[39] This was then distilled under vacuum into a suspension of lithium amide in 100 ml of liquid ammonia (prepared from 173 mg of lithium and 25 mg of $Fe(NO_3)_3 \cdot 6H_2O$), which was cooled with liquid nitrogen. The mixture was then placed in a bath at −40° and stirred for 30 min. One gram of 1,1-dimethoxybutan-3-one was then added and the reaction mixture was stirred at −40° for another 2 hr. Following neutralization with 2.4 g of NH_4Cl the ammonia was evaporated while 200 ml of ether was added, followed by 30 ml of H_2O. The ether and the aqueous layer were separated and the aqueous phase extracted continuously with ether for 4 hr. The combined ether extracts were evaporated and then dried further at 10^{-2} Torr to give 646 mg (50% yield) of ethyl 5,5-dimethoxy-3-methyl-3-hydroxyvalerate-*2*-^{13}*C* as a yellowish oil. The structure of this intermediate and the presence and location of the ^{13}C were confirmed by ^{1}H nmr. The ester acetal was hydrolyzed with a solution of 1 g of $Ba(OH)_2 \cdot 8H_2O$ in 12 ml of H_2O for 4 hr at 40°, the excess Ba^{2+} was then removed by addition of Dry Ice and after filtration the solution was evaporated to dryness under vacuum. The residue and 375 mg of $NaBH_3CN$[40] were dissolved in 5 ml of methanol containing a drop of methyl orange indicator, and 2 *N* HCl was added dropwise with stirring to maintain the red color of the indicator. After 15 min 35 ml of 0.1 *N* citrate buffer, pH 2.7, was added and stirring was continued for 48 hr at room temperature. Two milliliters of 5 *N* HCl were then added and the solution was saturated with NaCl and continuously extracted with ether for 5 hr. The ether extract was dried with Na_2SO_4 and evaporated to dryness. The residue was converted to the dibenzylethylenediamine salt of mevalonic-*2*-^{13}*C* acid by the method of Eggerer, *et al.*[7] The yield by titration was ~100% and as crystalline product (due to losses) 10.3%, mp 123°.

Mevalonic-*3'*-d_3 Acid.[22,23] To a mixture of 38 g of dihydropyran and 30 mg of *p*-toluenesulfonic acid was added slowly with cooling 18 g of acetic-d_4 acid. After 1 hr at room temperature, this mixture was diluted with an equal volume of ether and added dropwise with stirring and ice cooling to a Grignard solution prepared from 60.5 g of magnesium turnings and 181.5 g of freshly distilled allyl bromide in 750 ml of dry ether. The reaction was stirred for 1 hr at room temperature, then cooled and treated with ice and saturated NH_4Cl solution, and the aqueous phase was extracted four times with ether. The ether extract was washed with dilute $NaHCO_3$ solution and water, dried with Na_2SO_4, and distilled *in vacuo*. After removal of the volatile materials, the fraction distilling at 54–71° (10 mm) was collected and redistilled at atmospheric pressure (bp 151–155°) to give 23.0 g of methyldiallylcarbinol (63.3%). This material was dissolved in 150 ml of methanol, ozonized for 10 hours with ice cooling, and then treated in portions with 10 g of $NaBH_4$ with good cooling. Water was added and the mixture was washed through a column of 250 g of Dowex 50 H^+. The effluent and washings were evaporated in a rotary evaporator; methanol was added to the residue several times and evaporated again. In the last evaporation the temperature was raised to near 100°. The residue consisted of 12.4 g of 3-methylpentane-1,3,5-triol (50.6%). The product was dissolved in 80 ml of glacial acetic acid and added slowly with stirring and slight water cooling to 13.6 g of CrO_3 in 800 ml of glacial acetic acid and 17 ml of H_2O. The mixture was left for 15 hr at room temperature; 35 ml of methanol and 1.7 l. of water were then added and the solution was extracted continuously with ether for 2 days. The extract was evaporated on a rotary evaporator and benzene was added to the residue several times and evaporated again. The residue was taken up in 50% ethanol and titrated with 0.4 *N* $Ba(OH)_2$; the equivalent amount of dibenzylethylenediamine sulfate was added as a saturated aqueous solution and the barium sulfate was removed by centrifugation. After evaporation of the solution to dryness the residue was crystallized from methanol–ether to give 1.3 g of crude DBED–mevalonate (5.2%). Repeated recrystallization gave pure material of mp 124–126°.

Chanoclavine-I-17-*t*. Seventy milligrams of chanoclavine-I was dissolved in 1 ml of acetic anhydride at room temperature and the solution was immediately evaporated to dryness on a rotary evaporator. The residue was taken up in 2 ml of ethanol, evaporated again, and then chromatographed on a column of 10 g of Al_2O_3 (Brockmann) with chloroform containing 0.5% methanol as the developing solvent. The fractions containing *N*-acetylchanoclavine-I were combined and evaporated to give 51.3 mg (by colorimetry) of product. The latter was dissolved in 10 ml of anhydrous acetone and stirred with 490 mg of MnO_2[41] for 5 hr at

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Table IX. ^{1}H Nmr Spectral Data of Chanoclavine-I-aldehyde

Compd	C–CH_3	N–CH_3	4-CH_2, 5-CH	10-CH	CH_2–OH	9-HC=C—	Arom H	Indole H	O=CH
Chanoclavine-I	2.1 (br, s)	2.67 (s)	3.1–3.9 (m)	4.50 (m)	4.42 (br, s)	5.94 (m)	7–7.7 (m)	11.6 (m)	
Chanoclavine-I-aldehyde	2.08 (br, s)	2.51 (s)	2.8–3.6 (m)	4.40 (m)		6.75 (m)	6.8–7.7 (m)	11.7 (m)	9.74

room temperature. After filtration, the solution was evaporated and the residue crystallized from chloroform–hexane to give 48.8 mg of slightly impure *N*-acetylchanoclavine-I-aldehyde,[9] which was used without further purification in the next step.

The aldehyde (48.8 mg) was dissolved in 2 ml of tetrahydrofuran (freshly distilled over $NaBH_4$), and 10 μl of 1.25 *M* aqueous $NaBH_4$ solution was added, followed 30 min later by 100 mCi of tritiated $NaBH_4$ (16 Ci/mmol, 6.25 μmol). The tritiated material was added as a solid and the ampoule containing it was then rinsed with an aliquot of the reaction mixture and then with 0.1 ml of H_2O. The combined solutions were kept for 1.5 hr, when 100 μl of 1.25 *M* $NaBH_4$ (unlabeled) was added. After another hour 2 ml of H_2O were added, the mixture was extracted with ether, and the ether extract was dried over Na_2SO_4 and evaporated to dryness. The residue was chromatographed on a column of 20 g of Al_2O_3 (Brockmann), which was eluted first with chloroform and then with chloroform containing 0.5% methanol. As before, the fractions containing *N*-acetylchanoclavine-I were combined and evaporated to dryness to give 95 mCi of oily product which crystallized in the freezer. Thin-layer chromatography at this point indicated the presence of a single radioactive component, which coincided in R_f with the only van Urk-positive spot on the chromatograms and with authentic *N*-acetylchanoclavine-I. In view of the very poor yield in the next step, later preparations of chanoclavine-I-*17-t* involved the direct reduction of chanoclavine-I-aldehyde (see below) rather than the acetyl derivative by exactly the same technique.

A portion of the *N*-acetylchanoclavine-I-*17-t* (76 mCi = 1.67×10^{11} dpm) was heated with 1.5 ml of ethanol and 0.5 ml of 10 *N* aqueous KOH in an evacuated, sealed tube for 2 hr at 170°. The black reaction mixture was diluted with water and extracted with chloroform. Tlc of the extract showed that most of the radioactivity was still associated with unreacted *N*-acetylchanoclavine-I, some with a new less polar compound, and only very little with chanoclavine-I. To separate the latter from starting material, the chloroform solution was extracted with 2% aqueous succinic acid solution, the aqueous phase made alkaline with NH_4OH, and the alkaloid reextracted into ether. Thin-layer chromatography at this point showed about equal amounts of radioactivity associated with *N*-acetylchanoclavine-I and chanoclavine-I. The latter was then purified to radiochemical homogeneity by successive preparative layer chromatography on silica gel H (Merck), first with *tert*-butyl alcohol–$CHCl_3$ 1:3 in an ammonia atmosphere and second with $CHCl_3$–ethanol 1:1 as developing solvents. The final yield of chanoclavine-I-*17-t* was 5×10^8 dpm (0.3%).

Chanoclavine-I-aldehyde. Chanoclavine-I (32 mg), 450 mg of MnO_2,[41] and 150 ml of acetone were refluxed for 1.5 hr when tlc indicated that all of the chanoclavine-I had been reacted. The manganese dioxide was filtered off, the pale yellow solution evaporated to dryness, and the residue subjected to preparative layer chromatography (silica gel H, $CHCl_3$–methanol 7:3). The band at R_f 0.5 (blue-green color with *p*-dimethylaminobenzaldehyde) was eluted with methanol to give 24.5 mg (76%) of chanoclavine-I-aldehyde: average yields range from 50–80%; mp 119–123° (from $CHCl_3$–hexane); uv (ethanol) λ_{max} (log ϵ) 227 (4.57), 276 nm (3.91); ir absorptions ($CHCl_3$) 2.87 (N–H, medium), 5.92 μ (C=O strong); ORD (*c* 0.003, pyridine) $[\alpha]_{320}$ −52.6° (maximum, negative Cotton effect); mass spectrum *m/e* (composition, intensity) 254.1403 (100) (calcd for $C_{16}H_{18}N_2O$: 254.1419), 237 ($C_{16}H_{17}N_2$, 50), 236 ($C_{16}H_{16}N_2$, 54), 235 ($C_{16}H_{15}N_2$, 80), 194 ($C_{14}H_{12}N$, 48), 168 (50), 167 ($C_{12}H_9N$, 46), 155 ($C_{11}H_9N$, 54), 154 ($C_{11}H_8N$, 62); ^{1}H nmr spectrum (pyridine-d_5) the ^{1}H nmr data for chanoclavine-I-aldehyde and, for comparison, chanoclavine-I are shown in Table IX.

For the preparation of chanoclavine-I-aldehyde-*17-t* and -^{14}*C-17-t* the correspondingly labeled chanoclavine samples were oxidized as above. In the case of the double labeled material, chanoclavine-I of T/^{14}C = 3.50 gave aldehyde of T/^{14}C = 1.78, confirming that all the tritium was present at C-17.

Nonlabeled chanoclavine-I used as starting material was obtained in two ways. The first samples were isolated from the fermentation broth of *Claviceps paspali* strain Li 342/SE 156[11] and were a gift from Professor Gröger, Halle. Later, further quantities were prepared chemically from elymoclavine by conversion to *N*-methylsecoelymoclavine,[12] which was then demethylated with diethyl azodicarboxylate as described by Fehr[9] to give chanoclavine-I in about 20–30% overall yield.

Degradation of Elymoclavine. Degradations to determine the amount of tritium present at C-7 of elymoclavine were carried out as described earlier.[5]

General Techniques. ^{1}H nmr spectra were recorded on a Jeol MH 60, a Varian XL-100, or a Varian HR-220 nmr spectrometer using tetramethylsilane as internal standard, ir spectra on a Perkin-Elmer Infracord, uv spectra on a Beckman 124, and ORD spectra on a Cary 60 instrument. Low resolution mass spectra were obtained on a Hitachi RMU-6 and a CEC 2110 spectrometer. The latter was also used for the high resolution spectra. For quantitative isotope analyses, several slow scans of the molecular ion region were usually recorded at low electron voltage. Radioactivity determinations were done by liquid scintillation counting in a Beckman LS 100 scintillation counter, using PPO and dimethyl-POPOP in toluene as scintillator solution and internal standardization as the means of determining counting efficiencies. Radioactivity on chromatograms was located by scanning in a Packard Model 7401 radiochromatogram scanner. Indolic compounds on chromatograms were visualized by spraying with van Urk's spray reagent (1 g of *p*-dimethylaminobenzaldehyde in 10 ml of H_2O and 20 ml of concentrated HCl).

Calculation of Isotope Composition from Mass Spectral Data. The actual isotope composition of deuterium- and/or ^{13}C-labeled compounds was calculated from the intensities of the molecular ion and its isotope satellites of the labeled compound and the corresponding unlabeled compound, using Biemann's formulas[42] for the correction for natural isotope abundances. Biemann's formula for the correction in the presence of an M − 1 peak did not work for elymoclavine in experiments 1–4; it gave large negative values for some of the isotopic species, possibly because of the presence of deuterium in positions from which a hydrogen might be eliminated (C-7). This problem was not encountered in experiments 6 and 7. To overcome the difficulty, all peaks were first corrected for the natural ^{13}C, ^{15}N, and ^{18}O content, using the theoretical values. Correction for the P − 1 contribution then involved a series of approximations in which the ratio of (P − 1)/P was varied, starting from the observed I_{253}/I_{254} ratio, until the intensity at 253 (M − I) became 0. The values derived in this way may be only approximate, because the method of calculation includes the assumption that all peaks of the molecular ion region give a P − 1 peak in the same ratio, which may not be true in view of the presence of deuterium at C-7.

Estimations of the expected deuterium distributions in tetracyclic ergolines in experiments 1–4 are based on the observed distribution in chanoclavine-I from the same experiment, on the experimentally determined tritium retentions, and on the knowledge (from the deuterium distribution in the precursor) that all chanoclavine-I molecules containing any deuterium will carry deuterium at C-9. For the case of the simple exchange at C-9, the fraction of each species corresponding to the tritium retention is retained and the rest is converted to the P − 1 species. For example, in experiment 1 (53% tritium retention) 28.4% D_3 give 15.1% D_3 and 13.3% D_2, 12.4% D_2 produce 6.6% D_2 and 5.8% D_1, 1.8% D_1 give 1.0% D_1 and 0.8% D_0. Therefore, the predicted distribution in the tetracyclic ergolines is 15.1% D_3, 13.3% + 6.6% = 19.9% D_2, 5.8% + 1.0% = 6.8% D_1, and 57.4% + 0.8% = 58.2% D_0 species. To correct for the loss of one hydrogen from C-17, all the D_3 species in chanoclavine-I are converted to D_2 species. However, only half of the D_2 species will give rise to D_1 species whereas the other half remains D_2, because in the D_2 molecules only one of the two methylene hydrogens is replaced by deuterium and there is an almost equal chance of losing H or D. The D_1 species remain unchanged because they are only labeled at C-9. The resulting distribution

(42) K. Biemann, "Mass Spectrometry, Organic Chemical Applications," McGraw-Hill, New York, N. Y., 1962, pp 224–227.

is then used to correct for exchange or intermolecular transfer. Predicting the distribution for the intermolecular transfer involves the following process (example experiment 1): (a) remove one atom of D from all deuterated species to give 28.4% D_2 + 12.4% D_1 + 59.2% D_0 and 42.6% D which is being transferred, (b) exchange 47% of the deuterium which has been removed for H (the hydrogen being transferred is now 22.6% D and 77.4% H), (c) recombine this H/D mixture statistically with the mixture of acceptor molecules from a to give 28.4% × 22.6% = 6.4% D_3, 28.4% × 77.4% = 22.0% D_2, 12.4% × 22.6% = 2.8% D_2, 12.4% × 77.4% = 9.6% D_1, 59.2% × 22.6% = 13.4% D_1, and 59.2% × 77.4% = 45.8% D_0, or a predicted distribution of 6.4% D_3, 24.8% D_2, 23.0% D_1, and 45.8% D_0. This prediction is obviously only very approximate since it assumes that the final ratio of labeled to unlabeled chanoclavine-I molecules is the same as that throughout the experiment. The prediction of the deuterium distribution for the case of intermolecular transfer and loss of one hydrogen from C-17 involves the same process using the figures obtained after correcting chanoclavine-I for loss of one hydrogen from C-17. Alternatively, the figures for the intermolecular transfer case can be used and corrected for loss of one atom of deuterium, giving a somewhat different set of numbers. Obviously, the number of assumptions inherent in these estimations is too great to allow prediction of accurate figures and the range covered by these two modes of calculation is therefore given.

In experiments 6 and 7 the prediction of the range of double labeled molecules expected is based on the following consideration. The minimum value follows from the assumption that the isotope distribution in chanoclavine-I was constant throughout the experiment. Single labeled chanoclavine-I species consist of ^{13}C and D molecules in a ratio of 43:50, *i.e.*, in experiment 6 of 19.4% ^{13}C and 22.6% D. With a tritium retention of 65%, of the deuterium present 7.9% will exchange and the remaining 14.7% will statistically combine with 80.6% ^{12}C and 19.4% ^{13}C molecules to give 2.9% molecules containing ^{13}C and D. The other extreme follows from the assumption that all the labeled alkaloid is formed at a different time than unlabeled alkaloid from endogenous mevalonate. In this case there is no dilution of the precursor and therefore 65% of the deuterium of mevalonate (*i.e.*, 32.5% D) would be distributed between 57% ^{12}C and 43% ^{13}C molecules to give 43% × 32.5% = 14% molecules containing ^{13}C + D.

Acknowledgments. We are greatly indebted to Drs. A. Hofmann and H. Kobel, Sandoz AG, Basel, for generous supplies of elymoclavine and agroclavine and to Dr. D. Gröger, Halle (Saale), GDR, for providing both unlabeled and ^{14}C-labeled chanoclavine-I. Thanks are also due to the Purdue University Mass Spectrometry Center, particularly Dr. G. E. van Lear, Dr. G. Cooks, and Mr. W. Perry, for a large number of mass spectra, to Dr. John Grützner, Purdue, Dr. E. Wenkert and Mr. E. Hagaman, Indiana University, and Dr. K. Nakanishi, Columbia University, for high-resolution nmr spectra, and to the U. S. Public Health Service for financial support (Research Grants AM 11662 and AM 13555 and Research Career Development Award GM 42389 to H. G. F.).

Isolation, Gas Chromatography–Mass Spectrometry, and Structures of New Alkaloids from *Erythrina folkersii* Krukoff and Moldenke and *Erythrina salviiflora* Krukoff and Barneby[1]

David S. Millington, Douglas H. Steinman, and Kenneth L. Rinehart, Jr.*

Contribution from the Roger Adams Laboratory, University of Illinois, Urbana, Illinois 61801. *Received August 20, 1973*

Abstract: We have developed a method for routine analysis of the *Erythrina* alkaloids to facilitate chemotaxonomic studies on species of *Erythrina*. The crude alkaloid mixtures are resolved and identified as their trimsyl (trimethylsilyl) derivatives by combined gas chromatography–mass spectrometry. We report here the salient gas chromatographic and mass spectral characteristics of the trimsyl derivatives of alkaloids from *E. folkersii* Krukoff and Moldenke and *E. salviiflora* Krukoff and Barneby. We also describe the isolation of three new *Erythrina* alkaloids—erysoline (**5**), erythravine (**8**), and erysosalvine (**12**)—and the isolation of erysotine (**11**) and erysotinone (**17**) for the first time from a natural source. Two more new *Erythrina* alkaloids–erysoflorinone and erysosalvinone (**16** and **18**)—have been characterized by gas chromatography–mass spectrometry as their trimsyl derivatives.

The *Erythrina* alkaloids[2] have been the subject of active investigation in recent years with respect to biosynthesis,[3] identification of known and new alkaloids,[4] mass spectrometric behavior,[5] and total synthesis.[6] Our own efforts have been directed toward a

(1) Presented in part at the 19th Annual Conference on Mass Spectrometry and Allied Topics, Atlanta, Ga., May 1971, Paper F7, and at the 8th International Symposium on the Chemistry of Natural Products, IUPAC, New Delhi, India, Feb 1972, Abstract A31.

(2) Reviews: (a) L. Marion in "The Alkaloids," Vol. 2, R. H. F. Manske and H. L. Holmes, Ed., Academic Press, New York, N. Y., 1952, Chapter 14, pp 499–511; (b) V. Boekelheide, *ibid.*, R. H. F. Manske, Ed., Academic Press, New York, N. Y., 1960, Chapter 11, pp 201–227; (c) R. K. Hill, *ibid.*, Vol. 9, R. H. F. Manske, Ed., Academic Press, New York, N. Y., 1967, Chapter 12, pp 483–515; (d) H.-G. Boit, "Ergebnisse der Alkaloid-Chemie bis 1960," Akademie-Verlag, Berlin, 1961, Chapter 22, pp 383–401.

(3) (a) D. H. R. Barton, R. James, G. W. Kirby, D. W. Turner, and D. A. Widdowson, *J. Chem. Soc. C*, 1529 (1968); (b) D. H. R. Barton, R. B. Boar, and D. A. Widdowson, *ibid.*, 1208, 1213 (1970); (c) B. Franck and V. Teetz, *Angew. Chem.*, **83**, 409 (1971).

(4) (a) D. H. R. Barton, A. A. L. Gunatilaka, R. M. Letcher, A. M. F. T. Lobo, and D. A. Widdowson, *J. Chem. Soc., Perkin Trans. 1*, 874 (1973); (b) D. H. R. Barton, P. N. Jenkins, R. M. Letcher, D. A. Widdowson, E. Hough, and D. Rogers, *Chem. Commun.*, 391 (1970); (c) K. Ito, H. Furukawa, and H. Tanaka, *Chem. Pharm. Bull.*, **19**, 1509 (1971); (d) *Chem. Commun.*, 1076 (1970); (e) S. Ghosal, S. K. Dutta, and S. K. Bhattacharya, *J. Pharm. Sci.*, **61**, 1274 (1972); (f) S. Ghosal, A. Chakraborti, and R. S. Srivastava, *Phytochemistry*, **11**, 2101 (1972); (g) S. Ghosal, S. K. Majumdar, and A. Chakraborti, *Aust. J. Chem.*, **24**, 2733 (1971); (h) S. Ghosal, D. K. Ghosh, and S. K. Dutta, *Phytochemistry*, **9**, 2397 (1970); (i) R. M. Letcher, *J. Chem. Soc. C*, 652 (1971); (j) G. A. Miana, M. Ikram, F. Sultana, and M. I. Khan, *Lloydia*, **35**, 92 (1972); (k) H. Singh and A. Singh Chawla, *Experientia*, **25**, 785 (1969).

(5) R. B. Boar and D. A. Widdowson, *J. Chem. Soc. B*, 1591 (1970).

(6) (a) A. Mondon and M. Ehrhardt, *Tetrahedron Lett.*, 2557 (1966); (b) A. Mondon and P. R. Seidel, *Chem. Ber.*, **104**, 2937 (1971); (c) A. Mondon, *ibid.*, **104**, 2960 (1971); (d) A. Mondon, G. Aumann, and E. Oelrich, *ibid.*, **105**, 2025 (1972); (e) J. Kametani, T. Kohno, and K. Fukumoto, *Chem. Pharm. Bull*, **20**, 1678 (1972).

L4 ANSWER 4 OF 4 CAPLUS COPYRIGHT 2002 ACS DUPLICATE 2

AB The content of ergot alkaloids (ergovaline and **chanoclavine**), and their prodn. in Oct. 1996 and during the period May-Sept. 1997 was investigated in seventeen ecotypes of perennial ryegrass (Lolium perenne L.) and in nineteen ecotypes of meadow fescue (Festuca pratensis Huds.), naturally infected with **Neotyphodium** spp. The ecotypes were collected in the north-eastern part of the Czech Republic. In 1996, the content of ergovaline in the ecotypes of perennial ryegrass ranged from 0.cntdot.00 to 27.cntdot.73 .mu.g g-1 dry matter (DM) (one cut), and in 1997 from 0.cntdot.00 to 4.cntdot.65 .mu.g g-1 DM (five cuts). In meadow fescue, the content of ergovaline varied from 0.cntdot.00 to 0.cntdot.61 .mu.g g-1 DM (one cut) in 1996, and in 1997 from 0.cntdot.00 to 2.cntdot.31 .mu.g g-1 DM (five cuts). The content of **chanoclavine** (investigated in 1997 in four cuts only) in perennial ryegrass ranged between 0.cntdot.00 and 3.cntdot.39 .mu.g g-1 DM, and in meadow fescue between 0.cntdot.00 and 2.cntdot.26 .mu.g g-1 DM. Most ecotypes of L. perenne reacted to the high temp. and heavy rainfall in June and July of 1997 with an enhanced prodn. of ergovaline, whereas the content of **chanoclavine** was not changed. Such reaction to stress conditions was not obsd. in the ecotypes of F. pratensis. Large differences in the prodn. of both ergot alkaloids between different ecotypes of both plant species were obsd.

AN 2000:92846 CAPLUS

DN 132:276378

TI Concentration of ergot alkaloids in Czech ecotypes of Lolium perenne and Festuca pratensis

AU Cagas, B.; Flieger, M.; Olsovska, J.

CS OSEVA PRO Ltd. Grassland Research Station, Zubri, Czech Rep.

SO Grass and Forage Science (1999), 54(4), 365-370
CODEN: GFSCDW; ISSN: 0142-5242

PB Blackwell Science Ltd.

DT Journal

LA English

RE

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=> DIS HIST

     (FILE 'HOME' ENTERED AT 07:58:47 ON 17 JUL 2002)

     FILE 'REGISTRY' ENTERED AT 07:58:53 ON 17 JUL 2002
               E CHANOCLAVINE/CN
L1             1 S E3

     FILE 'CAPLUS, BIOSIS, USPATFULL, WPIDS, AGRICOLA' ENTERED AT 07:59:30 ON
     17 JUL 2002
L2           369 S L1 OR CHANOCLAVINE OR 2390-99-0
L3        564186 S FUNGUS
L4            75 S L2 AND L3
L5            46 S L2 (L) L3
L6            37 DUP REM L5 (9 DUPLICATES REMOVED)
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=> DIS HIST

     (FILE 'HOME' ENTERED AT 08:37:15 ON 22 AUG 2002)

     FILE 'CAPLUS, BIOSIS, USPATFULL, WPIDS, AGRICOLA' ENTERED AT 08:37:31 ON
     22 AUG 2002
L1           509 S NEOTYPHODIUM
L2           335 S CHANOCLAVINE
L3             7 S L1 AND L2
L4             4 DUP REM L3 (3 DUPLICATES REMOVED)

=>
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AU Beyerman, H. C.; van de Linde, A.; Henning., G. J.
CS Lab. Org. Chem., Tech. Hogeschool, Delft, Neth.
SO Chem. Weekblad (1963), 59(37), 508-9

Ergot alkaloids. XLII. New alkaloids from saprophytic culture of ergot fungus from Pennisetum typhoideum (P. glaucum)
AU Hofmann, A.; Brunner, R.; Kobel, H.; Brack, A.
CS Sandoz A.-G., Basel, Switz.
SO Helv. Chim. Acta (1957), 40, 1358-73
DT

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150. Neue Alkal ide aus der sapr phytischen Kultur des Mutterk rnpilzes von *Pennisetum typhoideum Rich.*

42. Mitteilung über Mutterkornalkaloide[1])

von **A. Hofmann, R. Brunner, H. Kobel** und **A. Brack.**

Herrn Prof. *T. Reichstein* zum 60. Geburtstag gewidmet.

(29. V. 57.)

In der 36. Mitteilung dieser Reihe[2]) wurde berichtet, dass ein Mutterkornpilz, den wir aus Sklerotien gezüchtet hatten, die auf der afrikanischen Kolbenhirse *Pennisetum typhoideum Rich.* gefunden worden waren, befähigt ist, bei der Züchtung *in vitro* Alkaloide zu bilden. Es konnten damals die zwei bereits bekannten Alkaloide Agroclavin und Elymoclavin, sowie ein neues Alkaloid, das wir Penniclavin nannten, aus Mycel und Filtrat von saprophytischen Kulturen dieses Pilzes isoliert werden.

Seither sind diese Untersuchungen fortgeführt worden. Dabei liess sich die Alkaloidausbeute wesentlich steigern. Ferner wurden vier neue Alkaloide gefunden, über deren Isolierung, Charakterisierung und Strukturaufklärung nachstehend berichtet wird[3]).

Die aus verschiedenem Sklerotienmaterial afrikanischer Herkunft isolierten Pilzstämme produzierten *in vitro* ganz unterschiedliche Mengen Alkaloide; sie waren offenbar genetisch nicht einheitlich. Wir führten daher unsere Infektionsversuche auf *Pennisetum*-Pflanzen im Gewächshaus fort, mit dem Erfolg, dass es gelang, den Pilz bis zum fertigen Sklerotienstadium auf der Wirtspflanze zu züchten. Das auf diese Weise erhaltene Sklerotien-Material erlaubte uns die Isolierung einer grösseren Anzahl von Pilzstämmen und gab uns die Möglichkeit einer Auswahl aus den verschiedenen Stammzüchtungen. Durch diese Auswahl kamen wir zu Stämmen, die bei der *in-vitro*-Kultur wesentlich höhere Alkaloidausbeuten lieferten.

Ferner wurden die Ernährungsbedingungen für die Kultur des Pilzes *in vitro* variiert, womit sich eine weitere Steigerung der Alkaloidproduktion erreichen liess. Während in den Versuchen, die in der 36. Mitteilung dieser Reihe beschrieben wurden, durchschnittliche Alkaloidausbeuten von ca. 50 mg/l Nährlösung und ca. 0,1% im Trockenmycel erzielt wurden, erhielten wir nun mit den neuen Pilzstämmen und der verbesserten Nährlösung bei grösseren Ansätzen

[1]) 41. Mitteilung, Helv. **39**, 1165 (1956).

[2]) *A. Stoll, A. Brack, H. Kobel, A. Hofmann & R. Brunner*, Helv. **37**, 1815 (1954).

[3]) Über einen Teil der Ergebnisse dieser Untersuchung wurde am 14. Internat. Kongress für reine und angewandte Chemie am 23. Juli 1955 in Zürich referiert. Ref. Bd. 14. Int. Chemie-Kongress Zürich, **1955**, 131.

regelmässig präparative Alkaloidausbeuten zwischen 1000 und 1500 mg/l Kulturfiltrat und bis 1,0% im Trockenmycel.

Im Laufe dieser Versuche zeigte sich ferner, dass die Bebrütung der Kulturen auf der Schüttelmaschine ungeeignet ist, da sie zu starker Schleimbildung führt, was die Isolierung der Alkaloide sehr erschwert. Standkulturen erwiesen sich vorteilhafter.

In der Tab. 1 sind die Alkaloid-Ausbeuten von 5 grösseren Standkultur-Ansätzen zusammengestellt.

Tabelle 1.

Ansatz	Stamm Nr.	Kulturfiltrat			Trockenmycel		
		Ernte-menge l	Präp. Ausbeute		Menge kg	Präp. Ausbeute	
			Gesamt-alkaloide g	mg/l		Gesamt-alkaloide g	% des Mycels
1	231	98	105,6	1077	1,92	12,6	0,65
2	231	100	159,5	1595	2,28	18,4	0,81
3	231	91	132,2	1454	2,54	27,4	1,08
4	233	86	120,8	1404	1,83	15,5	0,85
5	233	91,5	113,0	1234	1,60	15,0	0,94
Total der 5 Ansätze		466,5 l	631,1 g		10,17 kg	88,9 g	

Die Alkaloide wurden aus den Kulturansätzen auf die früher beschriebene Weise[2]) isoliert und aufgearbeitet. Nach der Abtrennung der Hauptmenge des Elymoclavins, das stets zusammen mit dem Agroclavin den überwiegenden Anteil der von unseren Pilzstämmen produzierten Alkaloide ausmacht, wurde das verbleibende Gemisch an der Aluminiumoxydsäule chromatographiert. Beginnend mit absolutem Chloroform, dem steigende Mengen Methanol zugesetzt wurden, liessen sich in der nachstehenden Reihenfolge einheitliche Alkaloidfraktionen eluieren.

Mit absolutem Chloroform wurde das von *M. Abe*[4]) in japanischem Grasmutterkorn entdeckte Agroclavin herausgelöst, dessen Vorkommen im *Pennisetum*-Mutterkorn wir bereits in der 36. Mitteilung dieser Reihe beschrieben haben.

Bei der Fortsetzung der Elution mit Chloroform, das 0,5% Methanol enthielt, zeigten sich zwei weitere Maxima, in denen zwei neue, ein Isomerenpaar bildende Alkaloide, die wir Isosetoclavin und Setoclavin genannt haben, enthalten waren.

Mit Chloroform/1% Methanol folgte ein bekanntes Alkaloid, das Elymoclavin, das von *M. Abe* und Mitarb.[5]) in japanischem Gras-

[4]) *M. Abe*, Ann. Rep. Takeda Res. Lab. **10**, 73 (1951); *M. Abe & S. Yamatodani*, J. Agr. Chem. Soc. Japan **28**, 501 (1954).

[5]) *M. Abe, T. Yamano, Y. Kozu & M. Kusumoto*, J. Agr. Chem. Soc. Japan **25**, 458 (1952); *M. Abe & S. Yamatodani*, ibid. **28**, 501 (1954); **29**, 346 (1955); *S. Yamatodani & M. Abe*, Bull. Agr. Chem. Soc. Japan **19**, 94 (1955).

mutterkorn gefunden wurde, und dessen Vorkommen im Mutterkornpilz der Kolbenhirse wir ebenfalls bereits beschrieben haben.

Anschliessend eluierte Chloroform, das 2% Methanol enthielt, wieder ein neues Alkaloid, dem wir den Namen Chanoclavin gegeben haben.

Schliesslich zeigten sich beim Eluieren mit Chloroform/3% Methanol wieder zwei Maxima, die zwei Alkaloiden entsprachen, die ein Isomerenpaar bilden. Das eine war neu. Es wurde als Isopenniclavin bezeichnet. Das andere war Penniclavin, dessen Isolierung schon in der 36. Mitteilung beschrieben wurde.

Die neuen Alkaloide.

Setoclavin und Isosetoclavin. Die Werte der Elementaranalyse und der acidimetrischen Titration ergaben für beide Alkaloide die Bruttoformel $C_{16}H_{18}ON_2$. Beide besitzen eine C·CH_3-Gruppe und zwei aktive Wasserstoffatome. Die UV.-Spektren von Setoclavin und Isosetoclavin sind gleich und identisch mit dem der Lysergsäure und Isolysergsäure (Fig. 1). Die IR.-Spektren (Fig. 2), die durch starke Banden im Gebiet der Absorption der OH- und NH-Gruppe (2700 bis 3500 cm^{-1}) gekennzeichnet sind, weisen gewisse Unterschiede auf,

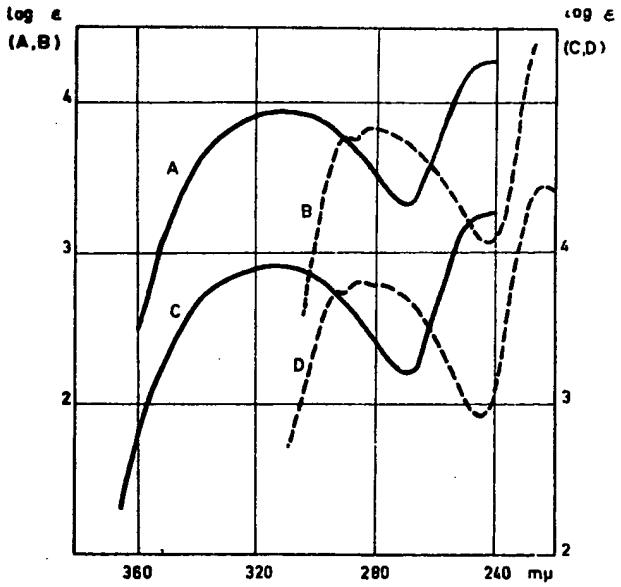


Fig. 1.
UV.-Spektren in Äthanol.
A: Lysergsäure, Isolysergsäure.
B: Dihydro-lysergsäure.
C: Setoclavin, Isosetoclavin, Penniclavin, Isopenniclavin.
D: Chanoclavin.

wie sie zwischen Stereoisomeren beobachtet werden. Die beiden Alkaloide stimmen auch in den Farbreaktionen überein, die von denen der Lysergsäure-Derivate stark verschieden sind. Mit *Keller*-Reagens wird

eine grüne Färbung erhalten. Bei der Farbreaktion nach *Van Urk* entsteht eine blasse, grüngelbe Färbung. Charakteristisch ist die intensive, reinblaue Farbe, mit der sich die beiden Alkaloide in konz. Schwefelsäure auflösen. Setoclavin und Isosetoclavin sind sehr säureempfindlich. Mineralsaure Lösungen verfärben sich rasch unter Bilddung dunkler Zersetzungsprodukte.

Setoclavin, das an der Aluminiumoxydsäule etwas stärker haftet als Isosetoclavin, kristallisiert leicht aus allen gebräuchlichen Lösungsmitteln, besonders schön aus Methanol oder Aceton, woraus es sich in dicken, kristallösungsmittelfreien Prismen abscheidet. Es schmilzt unter Zersetzung bei 229–234°[6]) und besitzt in Pyridin ein spez. Drehvermögen $[\alpha]_D^{20} = +174°$.

Isosetoclavin ist wie das Setoclavin sehr kristallisationsfreudig. Aus Methanol werden massive, kristallösungsmittelfreie Polyeder erhalten, die bei 234–237° unter Zersetzung schmelzen. Das spez. Drehvermögen in Pyridin beträgt $[\alpha]_D^{20} = +107°$.

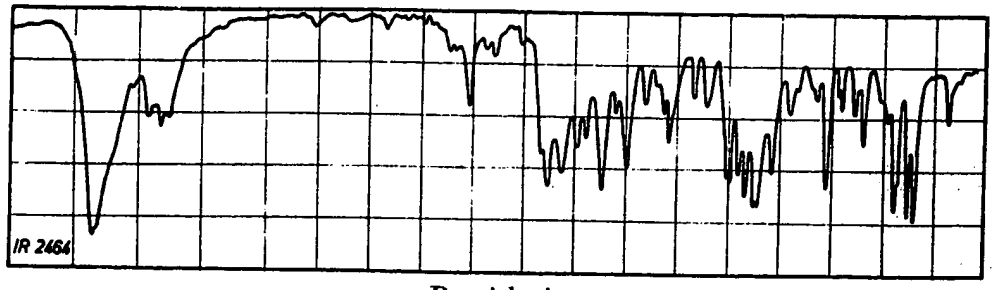
Dieses Alkaloid ist inzwischen von *M. Abe* und Mitarb.[7]) auch aus saprophytischen Kulturen eines Mutterkornpilzes isoliert worden, der auf *Agropyrum semicostatum Nees*, *Trisetum bifidum Ohwi*, *Festuca rubra L.* und anderen japanischen Gräsern wächst.

Chanoclavin. Dieses Alkaloid kristallisiert aus Methanol oder Aceton in dicken Prismen und Polyedern, die kein Kristallösungsmittel enthalten, und die bei 220–222° unter Zersetzung schmelzen. Es besitzt die Bruttoformel $C_{16}H_{20}ON_2$. Das spez. Drehvermögen $[\alpha]_D^{20} = -240°$ (in Pyridin) ist höher als das der übrigen aus dem *Pennisetum*-Pilz isolierten Alkaloide. Chanoclavin zeigt bei der Bestimmung nach *Kuhn-Roth* 1 C·CH_3-Gruppe an. Es ist stärker basisch als alle übrigen Mutterkornalkaloide ($pK_b = 5{,}80$). Das UV.-Spektrum (Fig. 1) ist nahezu identisch mit dem der Dihydro-lysergsäure. Das IR.-Spektrum (Fig. 3) zeigt die charakteristische Absorption der Indolderivate bei 1600–1650 cm^{-1} und starke Banden im Gebiet der OH- und NH-Absorption. Chanoclavin gibt bei der *Keller*'schen und der *Van Urk*'schen Farbreaktion die gleichen violettblauen Färbungen wie die Dihydro-lysergsäure und wie Agroclavin und Elymoclavin.

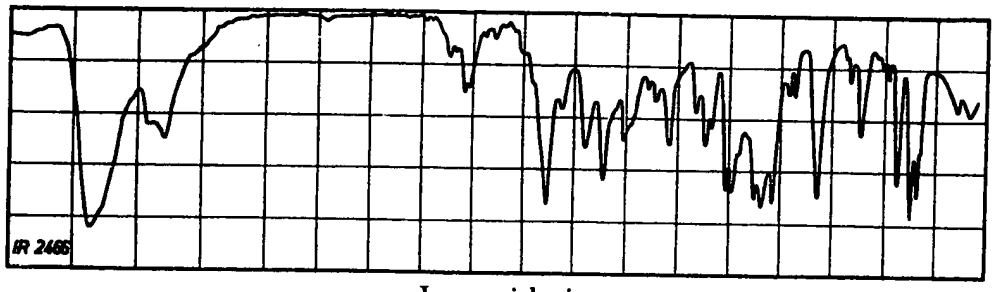
Isopenniclavin. Dieses Alkaloid, das an der Aluminiumoxydsäule etwas weniger stark haftet als Penniclavin, besitzt wie dieses die Bruttoformel $C_{16}H_{18}O_2N_2$. Es kristallisiert aus den üblichen organischen Lösungsmitteln, aber auch aus Wasser, aus dem es sich in 6-eckigen, kristallösungsmittel-freien Platten abscheidet, die bei 163 bis 165° unter Zersetzung schmelzen. Der spez. Drehwert $[\alpha]_D^{20}$ in Pyridin beträgt + 146°. Das UV.-Spektrum des Isopenniclavins (Fig. 1) ist

[6]) Alle Smp. dieser Arbeit wurden auf dem *Kofler*-Block bestimmt.

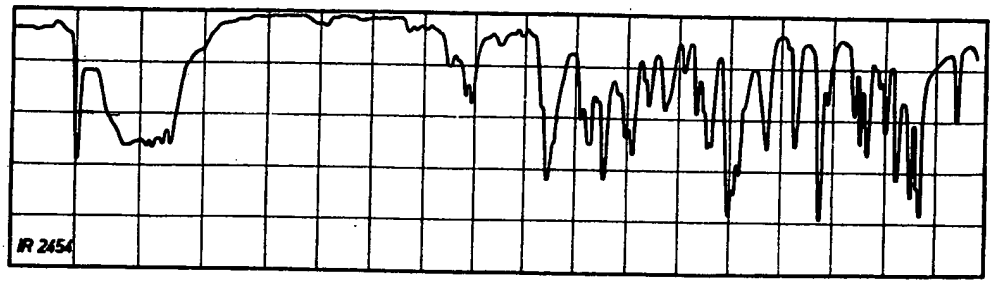
[7]) *M. Abe, S. Yamatodani, T. Yamano & M. Kusumoto*, Bull. Agr. Chem. Soc. Japan **20**, 59 (1956).



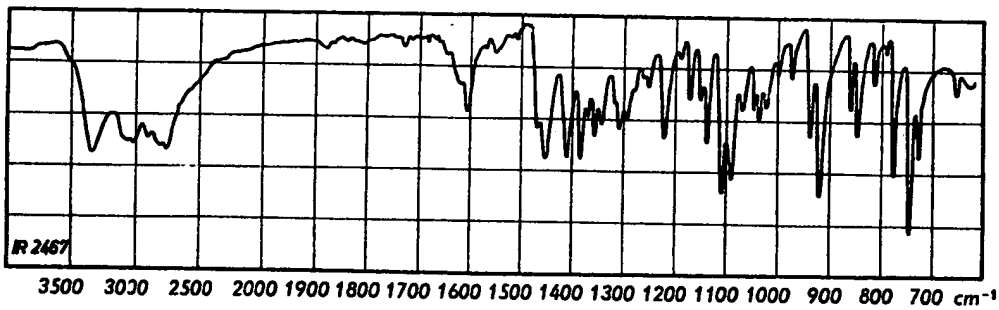
Penniclavin.



Isopenniclavin.



Setoclavin.

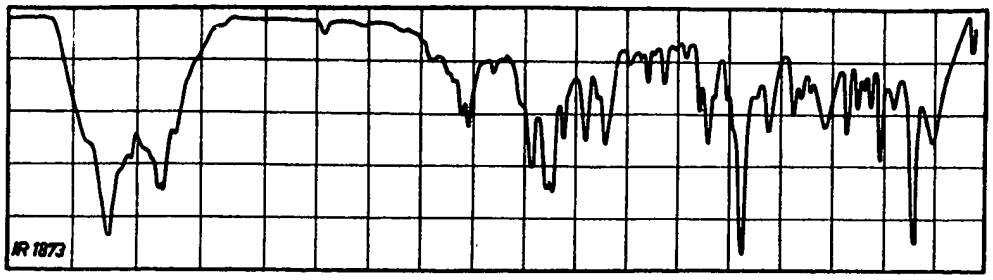


Isosetoclavin.

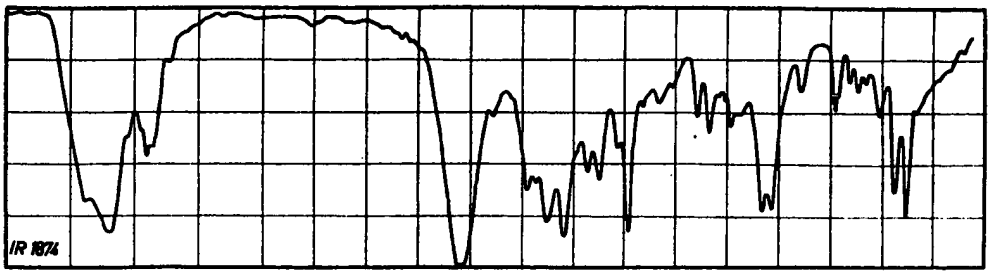
Fig. 2.

IR.-Spektren in KBr.

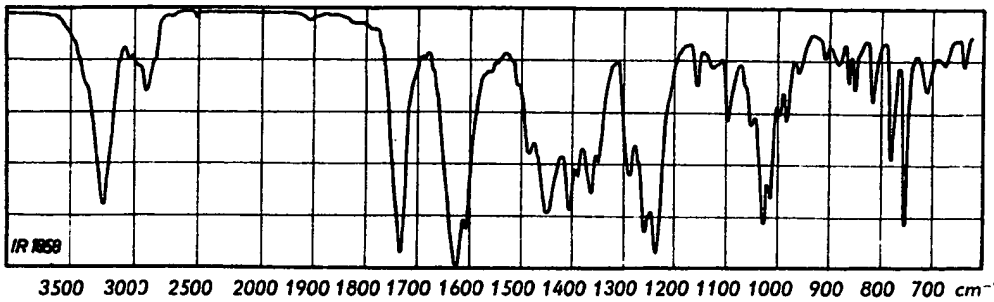
wie das des Penniclavins identisch mit dem der Lysergsäure und Isolysergsäure. Im IR.-Spektrum (Fig. 2), das durch starke Banden im Gebiet der OH- und NH-Absorption charakterisiert ist, stimmt das Isopenniclavin weitgehend mit dem Penniclavin überein. Isopenniclavin gibt die gleichen Farbreaktionen wie das Penniclavin, wie sie weiter oben beim Setoclavin und Isosetoclavin beschrieben wurden, und die von denen der Lysergsäure- und Dihydro-lysergsäure-Derivate stark verschieden sind.



Chanoclavin.



N-Acetylchanoclavin.



O,N-Diacetylchanoclavin.

Fig. 3.
IR.-Spektren in KBr.

In der Tab. 2 sind die wichtigsten Daten der 4 neuen Alkaloide mit denen der bereits früher beschriebenen, aus dem *Pennisetum*-Pilz isolierten Alkaloide zusammengestellt.

Tabelle 2.

Alkaloide aus der saprophytischen Kultur des Mutterkornpilzes von *Pennisetum typhoideum Rich.*

Name	Bruttoformel	Smp.[6]) (unter Zers.)	$[\alpha]_D^{20}$ (Pyridin)	*Keller*'sche Farbreaktion
Agroclavin . . .	$C_{16}H_{18}N_2$	210–212°	– 183°	violettblau
Elymoclavin . .	$C_{16}H_{18}ON_2$	245–247°	– 152°	violettblau
Chanoclavin . .	$C_{16}H_{20}ON_2$	220–222°	– 240°	violettblau
Setoclavin . . .	$C_{16}H_{18}ON_2$	229–234°	+ 174°	gelbgrün
Isosetoclavin . .	$C_{16}H_{18}ON_2$	234–237°	+ 107°	gelbgrün
P nniclavin . .	$C_{16}H_{18}O_2N_2$	222–225°	+ 153°	gelbgrün
Isopenniclavin .	$C_{16}H_{18}O_2N_2$	163–165°	+ 146°	gelbgrün

Die Strukturen von Agroclavin und Elymoclavin sind von *M. Abe et al.*[4])[5]) aufgeklärt worden. Sie werden durch die Formeln I und II wiedergegeben.

Die Konstitution von Setoclavin, Isosetoclavin, Penniclavin und Isopenniclavin.

Aus den UV.-Spektren dieser 4 Alkaloide (Fig. 1), die alle gleich und identisch mit dem der Lysergsäure und Isolysergsäure sind, konnte geschlossen werden, dass diesen Alkaloiden das gleiche chromophore System, das $\Delta^{9,10}$-Ergolen, zugrunde liegt. Die Bruttoformeln, die alle die gleiche Anzahl Kohlenstoffatome wie die Lysergsäure aufweisen, deuteten darauf hin, dass das Ergolen-Ringsystem in diesen zwei Alkaloidpaaren, gleich wie in der Lysergsäure, in Stellung 6 am Stickstoff eine Methylgruppe trägt, und dass auch das dem Carboxyl der Lysergsäure entsprechende C-Atom in Stellung 8 noch vorhanden ist. Auch die biogenetische Verwandtschaft dieser Substanzen rechtfertigte eine solche Annahme.

Die Natur des Sauerstoffatoms im Setoclavin und Isosetoclavin liess sich aus der Anzahl der aktiven Wasserstoffatome ableiten. Es wurden zwei aktive H-Atome gefunden, von denen eines auf die NH-Gruppe des Indols entfällt, das andere somit als alkoholisches Hydroxyl vorliegen muss. Damit stimmt die starke IR.-Absorption im Gebiet der OH-Frequenz überein (Fig. 2). Diese Hydroxylgruppe lässt sich nicht acetylieren. Sie wird offenbar sehr leicht abgespalten, unter Ausbildung einer zweiten Kohlenstoffdoppelbindung im Ring D des Ergolensystems. Solche Verbindungen sind aber nicht stabil; sie zersetzen sich zu dunklen Produkten[8]). Die oben erwähnte grosse Säureempfindlichkeit dieses Alkaloidpaares ist auf die leichte Abspaltbarkeit des Hydroxyls zurückzuführen, das damit als tertiäre OH-Gruppe charakterisiert ist. Im $\Delta^{9,10}$-Ergolen, das in 8-Stellung substituiert ist, kann aber ein tertiäres Hydroxyl nur am C-8 plaziert werden. Daraus ergeben sich für Setoclavin und Isosetoclavin die Strukturformeln III und IV.

Aus biogenetischen Gründen durfte angenommen werden, dass Setoclavin und Isosetoclavin am C-5 die gleiche Konfiguration besitzen, denn alle bisher aufgefundenen Mutterkornalkaloide stimmen konfigurativ an diesem Asymmetriezentrum überein. Es war daher wahrscheinlich, dass sich Setoclavin und Isosetoclavin am anderen asymmetrischen C-Atom unterscheiden, also Epimere am C-8 sind wie Lysergsäure und Isolysergsäure. Das liess sich beweisen, als es gelang Agroclavin (I), an dem die Konfiguration am C-5 stabil und gleich wie in der Lysergsäure ist, durch oxydative Hydroxylierung in ein Gemisch von Setoclavin und Isosetoclavin überzuführen.

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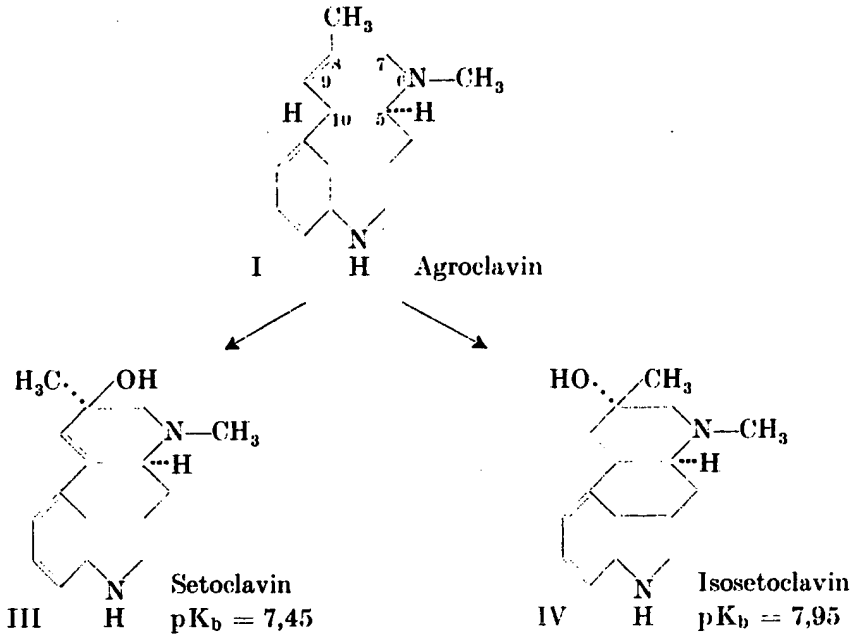
[8]) *A. Stoll, A. Hofmann & F. Troxler*, Helv. **32**, 506 (1949).

S. Yamatodani & M. Abe[9]) erhielten bei der Oxydation von Agroclavin mit Kaliumdichromat in verdünnter Schwefelsäure eine Substanz, die mit Triseclavin identisch war, einem Alkaloid, das *M. Abe et al*[10]) im Mutterkorn von japanischen Gräsern, wie *Elymus mollis Trin.*, *Trisetum bifidum Ohwi*, und in daraus bereiteten saprophytischen Kulturen gefunden hatten. Wir haben diese Oxydation, zu der die japanischen Autoren keine experimentellen Angaben machen,



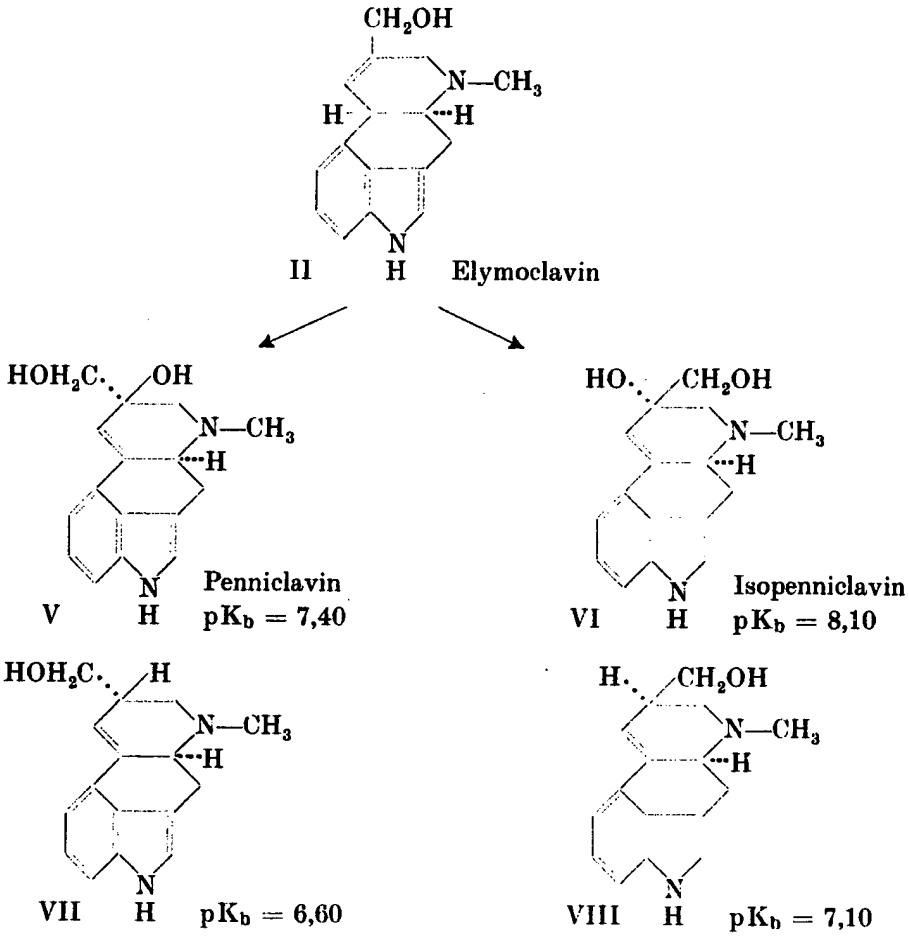
wiederholt und haben dabei aus reinstem Agroclavin ein Alkaloidgemisch, bestehend aus Setoclavin und Isosetoclavin, erhalten, aus dem sich die reinen, kristallisierten Komponenten mit einer Gesamtausbeute von 60% abtrennen liessen. *M. Abe et al.* geben für Triseclavin, dem sie die gleiche Struktur zuschrieben, wie wir sie für Setoclavin und Isosetoclavin vorgeschlagen haben, die spez. Drehung $[\alpha]_D^{18} = +137^0$ in Pyridin an. Dieser Wert liegt ziemlich genau in der Mitte von dem des Setoclavins und Isosetoclavins, so dass vermutet werden muss, dass es sich bei dem als Triseclavin beschriebenen Alkaloid um ein Mischkristallisat von Setoclavin und Isosetoclavin gehandelt hat.

Beim Penniclavin und Isopenniclavin, die zwei Sauerstoffatome enthalten, führte die Oxydation mit Perjodat zur Aufklärung ihrer Struktur. Beide Isomere spalten mit diesem Reagens Formaldehyd ab, womit die Gruppierung $-CHOH \cdot CH_2OH$ nachgewiesen ist. Eine Glykolgruppierung mit einem primären Hydroxyl lässt sich aber an dem für Penniclavin und Isopenniclavin postulierten Ergolen-Gerüst nur

[9]) Bull. Agr. Chem. Soc. Japan **19**, 94 (1955).

[10]) *M. Abe, S. Yamatodani, T. Yamano & M. Kusumoto*, Bull. Agr. Chem. Soc. Japan **19**, 92 (1955).

in 8-Stellung anbringen. Damit ergeben sich für dieses Isomerenpaar die Strukturformeln V und VI.



Diese wurden bestätigt, als es *S. Yamatodani & M. Abe*[9]) gelang, Elymoclavin durch Oxydation mit Dichromat in Penniclavin überzuführen. Bei der Nacharbeitung dieses Versuches erhielten wir aus Elymoclavin, allerdings nur in sehr geringer Ausbeute, ein Gemisch von Penniclavin und Isopenniclavin, aus dem die beiden Isomeren in reiner Form gewonnen werden konnten.

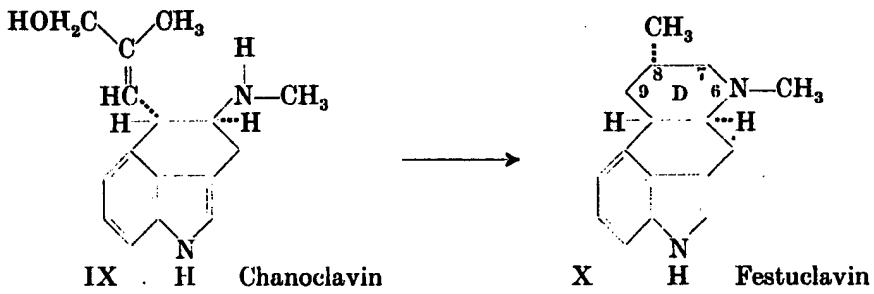
Das den beiden Isomerenpaaren Setoclavin-Isosetoclavin und Penniclavin-Isopenniclavin gemeinsame Strukturmerkmal, durch das sich diese Alkaloide von den übrigen bisher bekannten Mutterkornalkaloiden unterscheiden, ist die tertiäre Hydroxylgruppe am C-8. Diese ist für die grosse Säureempfindlichkeit, für die unterschiedlichen Farbreaktionen und die für Mutterkornalkaloide ungewohnten Drehwerte verantwortlich.

Durch den Eintritt einer Hydroxylgruppe an Stelle eines H-Atoms am asymmetrischen C-Atom 8, wird dieses ursprünglich sehr ungleichmässig belastete Asymmetriezentrum besser ausgewogen, was zur Folge hat, dass nun beide Epimere ähnliche Drehwerte aufweisen.

Man konnte daher bei diesen Alkaloidpaaren die konfigurative Zuordnung nicht mehr allein an Hand des Drehwertes (schwach positiver oder negativer Drehwert = D-Lysergsäure-Reihe, hoher positiver Drehwert = D-Isolysergsäure-Reihe) treffen. Es wurden daher noch andere geeignete Kriterien herangezogen. Aus dem Vergleich der pK_b-Werte und des adsorptiven Verhaltens der neuen Alkaloidpaarlinge mit den strukturell sehr nahestehenden Alkoholen D-Lysergol (VII) und D-Isolysergol (VIII), die aus D-Lysergsäure bzw. D-Isolysergsäure leicht zugänglich sind[11]), ergab sich die in den Bezeichnungen der Epimeren vorweggenommene konfigurative Zuordnung. D-Lysergol, Setoclavin und Penniclavin sind stärker basisch und haften an der Aluminiumoxydsäule besser als die entsprechenden Isoverbindungen.

Die Konstitution des Chanoclavins.

Dieses Alkaloid wurde so benannt, weil in ihm der Ring D des Ergolensystems zu einer offenen Kette aufgespalten ist. Während allen bisher bekannten Mutterkornalkaloiden das tetrazyklische Ergolen- oder Ergolin-Gerüst zugrunde liegt, leitet sich das Chanoclavin vom trizyklischen 1,3,4,5-Tetrahydro-benz(c,d)indol ab.



IX H Chanoclavin X H Festuclavin

Chanoclavin lässt sich zum Unterschied von den Alkaloiden der Setoclavin-Penniclavin-Gruppe sehr leicht mit Essigsäureanhydrid in Pyridin acetylieren. Dabei treten zwei Acetylgruppen in die Molekel ein. Diacetyl-chanoclavin ist nicht mehr basisch, was zeigt, dass das basische Stickstoffatom des Chanoclavins acetylierbar, also primär oder sekundär ist. Die zweite Acetylgruppe sitzt an einer leicht veresterbaren, also primären Hydroxylgruppe. Im IR.-Spektrum des Diacetyl-chanoclavins (Fig. 3) ist bei 1630 cm^{-1} die ausgeprägte Bande der Säureamid-Gruppe, bei 1740 cm^{-1} die charakteristische Absorption der Acetoxy-Gruppe sichtbar. Diese lässt sich alkalisch leicht verseifen, wobei man das Monoacetyl-chanoclavin erhält, in dem, wie aus dem IR.-Spektrum (Fig. 3) ersichtlich, die Acetamid-Gruppe noch vorhanden ist. Die Hydrolyse dieser Gruppe erfordert sehr energische Bedingungen, unter denen der grösste Teil der Substanz zerstört wird, wobei Chanoclavin nur in sehr geringer Ausbeute zurückgewonnen werden kann.

[11]) *A. Stoll, A. Hofmann & W. Schlientz*, Helv. **32**, 1947 (1949).

Chanoclavin besitzt die gleiche Anzahl C-Atome wie die Lysergsäure und die übrigen *Pennisetum*-Alkaloide, was gegen eine Formulierung als 6-Nor-Verbindung mit intaktem Ring D spricht. Als andere mögliche Variante mit acetylierbarem Stickstoff bleibt nur noch eine Formel, in welcher der Ring D des Ergolinsystems zwischen dem Stickstoff und dem C-Atom 7 geöffnet ist[12]). Die Bruttoformel des Chanoclavins verlangt bei geöffnetem Ring D in der Kohlenstoffseitenkette eine Doppelbindung. Diese darf, wie das UV.-Spektrum zeigt, nicht mit dem Indolsystem konjugiert sein. Es kommt somit nur die 7–8- oder die 8–9-Stellung in Frage. Da Chanoclavin eine C·CH_3-Gruppe aufweist, bleibt für die isolierte Kohlenstoffdoppelbindung nur noch die $\Delta^{8,9}$-Lage übrig. Alle diese Befunde werden durch die Strukturformel IX des Chanoclavins zum Ausdruck gebracht.

Auch die Konfiguration an den beiden asymmetrischen C-Atomen 5 und 10 des Chanoclavins liess sich abklären. Beim Versuch, die isolierte Doppelbindung des Chanoclavins selektiv zu hydrieren, wurde in geringer Menge Festuclavin[13]) (X) gebildet, das konfigurativ mit der Dihydro-lysergsäure-Reihe verknüpft ist. Dem Chanoclavin kommt also die im Formelbild IX zum Ausdruck gebrachte Konfiguration der Dihydro-lysergsäure(I)-Reihe[14]) zu. Die Substituenten an den beiden Asymmetriezentren sind in Trans-Stellung angeordnet.

Experimenteller Teil[6]).

1. Beispiel eines Züchtungsansatzes (Ansatz 2, Tab. I). Es wurde eine Nährlösung von folgender Zusammensetzung verwendet: Saccharose 100,0 g, Ammoniumsuccinat 10,1 g, $Ca(NO_3)_2$ 1,0 g, KH_2PO_4 0,25 g, $MgSO_4,7H_2O$ 0,25 g, KCl 0,125 g, $FeSO_4,7H_2O$ 8,34 mg, $ZnSO_4,7H_2O$ 3,44 mg, Leitungswasser ad 1000 cm³. 122 l dieser Nährlösung mit dem pH = 5,2 wurden in 500-cm³-Portionen in 1,6-l-*Fernbach*-Kolben abgefüllt, im Autoklaven sterilisiert und auf gleiche Art und Weise mit einer Sporensuspension des Pilzstammes Nr. 231 beimpft und bebrütet wie früher[2]) beschrieben.

Nach 27 Tagen erntete man die Kulturen ab. Es resultierten 100 l Kulturfiltrat mit einem Alkaloidgehalt von 1680 mg/l und 2,28 kg Trockenmycel mit einem Alkaloidgehalt von 1,08% (Gehaltsbestimmungen kolorimetrisch, auf Mol.-Gew. 250 berechnet[2]).

2. Isolierung der Alkaloide. Als Beispiel für die Aufarbeitung unserer Züchtungsansätze beschreiben wir nachstehend die Extraktion und chromatographische Aufteilung der Alkaloide aus dem Kulturfiltrat des unter 1. beschriebenen Versuches.

100 l Kulturfiltrat wurden nach Zusatz von 1 kg Soda einmal mit 50 l, dann noch fünfmal mit je 30 l Chloroform/Isopropylalkohol 3:1 ausgerührt. Die vereinigten Extrakte lieferten nach dem Eindampfen im Vakuum 248 g dunklen, öligen Rückstand.

Aus diesem kristallisierten nach dem Aufnehmen in 1,2 l Methanol 75,2 g rohes Alkaloid, aus dem durch Umkristallisieren aus Methanol 65,1 g reines Elymoclavin mit den früher beschriebenen Eigenschaften[2]) erhalten wurden.

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[12]) An dieser Stelle wird der Ring D geöffnet, wenn die Lysergsäure oder die Dihydro-lysergsäure mit Essigsäureanhydrid erhitzt wird (*A. Stoll, A. Hofmann & F. Troxler*, Helv. **32**, 506 (1949)).

[13]) *S. Yamatodani & M. Abe*, Bull. Agr. Chem. Soc. Japan **20**, 95 (1956).

[14]) *A. Stoll, Th. Petrzilka, J. Rutschmann, A. Hofmann & Hs. H. Günthard*, Helv. **37**, 2039 (1954).

Der Eindampfrückstand der vereinigten Elymoclavin-Mutterlaugen wurde in abs. Chloroform gelöst und an einer Säule aus 10 kg Aluminiumoxyd (*Brockmann*) chromatographiert. Beim Entwickeln mit dem gleichen Lösungsmittel wurden zuerst 42 g alkaloidfreies Öl und anschliessend 70,7 g Agroclavin eluiert. Durch Umkristallisieren aus Essigester erhielt man 69,4 g reines Alkaloid mit den früher angegebenen Daten[2]).

Mit Chloroform/0,5% Methanol liessen sich hierauf 7,0 g eines Alkaloidgemisches eluieren, das nochmals an der 100fachen Menge Aluminiumoxyd chromatographiert wurde. Dabei zeigten sich im Durchlaufchromatogramm beim Entwickeln mit Chloroform/0,5% Methanol zwei Maxima. Aus dem leichter eluierbaren Anteil (2,5 g) liessen sich durch Umkristallisieren aus Methanol 1,9 g reines Isosetoclavin gewinnen. Die Eluatrückstände der langsamer wandernden Zone wogen 2,9 g. Daraus wurden durch Umkristallisieren, zuerst aus Aceton, dann aus Methanol, 2,1 g reines Setoclavin erhalten.

Bei der Weiterentwicklung des Hauptchromatogrammes mit Chloroform/1% Methanol wurden 5,1 g Alkaloid herausgelöst, aus dem sich durch Umkristallisieren aus Methanol 4,8 g reines Elymoclavin gewinnen liessen.

Nach der Erhöhung des Methanolgehaltes im Chloroform auf 2% wurde eine Alkaloidfraktion eluiert (3,3 g), welche vorerst nur schlecht kristallisierte. Sie wurde zur Reinigung in wässeriger Weinsäure gelöst und nach der Vorextraktion dieser Lösung mit Äther, mit Soda wieder freigesetzt und in Äther aufgenommen. Der Ätherrückstand kristallisierte nun leicht aus Aceton. Durch Umkristallisieren aus diesem Lösungsmittel und anschliessend aus Methanol liessen sich 0,6 g reines Chanoclavin gewinnen.

Mit Chloroform/3% Methanol konnte schliesslich eine letzte Alkaloidfraktion herausgelöst werden (7,4 g), die erneut an einer Säule aus 740 g Aluminiumoxyd chromatographiert wurde. Beim Entwickeln mit Chloroform/3% Methanol bildeten sich zwei Hauptzonen. Die rascher wandernde enthielt 1,2 g einer Alkaloidfraktion, aus der sich durch mehrmaliges Umkristallisieren aus Essigester schliesslich 0,11 g reines Isopenniclavin gewinnen liessen. Aus der besser haftenden Alkaloidfraktion (5,8 g) konnten durch mehrmaliges Umkristallisieren zuerst aus Aceton, dann aus Methanol 1,9 g reines Penniclavin abgetrennt werden.

Zusammenfassung. Ausbeute an kristallisierten, einheitlichen Alkaloiden aus dem Kulturfiltrat, Ansatz 2, Stamm Nr. 231:

Agroclavin	69,4 g	Chanoclavin . . .	0,6 g
Elymoclavin . . .	69,9 g	Isopenniclavin . .	0,1 g
Isosetoclavin . . .	1,9 g	Penniclavin . . .	1,9 g
Setoclavin	2,1 g	Total	145,9 g

In den verschiedenen Kristallisationsmutterlaugen verblieben insgesamt 13,6 g amorphe Alkaloide, so dass die Totalausbeute an präparativ isolierten Alkaloiden 159,5 g beträgt, entsprechend 95% des kolorimetrisch im Kulturfiltrat ermittelten Gehaltes (168,0 g).

Aus dem Trockenmycel des Ansatzes 2 (2,28 kg) wurden die Alkaloide auf die früher beschriebene Weise[2]) extrahiert und, wie vorstehend beschrieben, an der Aluminiumoxydsäule chromatographiert. Dabei wurden folgende Ausbeuten erzielt:

Agroclavin kryst.	7,37 g
Agroclavin-Mutterlaugenrückstand	0,86 g
Elymoclavin kryst.	7,29 g
Elymoclavin-Mutterlaugenrückstand.	0,70 g
Setoclavin/Isosetoclavin-Fraktion	0,82 g
Penniclavin/Isopenniclavin-Fraktion.	1,26 g
Amorphe Restfraktion	0,15 g
Total	18,45 g

Das entspricht einer Gesamtalkaloidausbeute von 0,81%. Es wurden die gleichen Alkaloide in annähernd dem gleichen Mengenverhältnis wie aus dem Kulturfiltrat isoliert.

Chanoclavin, das nur in sehr geringer Menge vorkommt, konnte in diesem verhältnismässig kleinen Ansatz nicht gefasst werden.

Auch die übrigen von uns gezüchteten Stämme des *Pennisetum*-Pilzes produzierten Alkaloidgemische ähnlicher Zusammensetzung. Agroclavin und Elymoclavin waren stets die Hauptalkaloide, während die Mengenverhältnisse der einzelnen Nebenalkaloide beträchtlich variierten.

3. Beschreibung der einzelnen Alkaloide. a) Setoclavin. Kristallisiert aus Aceton oder Methanol in massiven, kristallösungsmittel-freien Prismen. Smp. 229—234°. Es löst sich bei Siedehitze in 40 Teilen der genannten Lösungsmittel, in 50 Teilen Essigester, in 40 Teilen Chloroform oder in 15 Teilen Dioxan. In Wasser ist das Alkaloid schwer löslich. $[\alpha]_D^{20} = +174^0$ ($\pm$ 2°), $[\alpha]_{5461}^{20} = +232^0$ ($\pm$ 3°) (c = 1,1 in Pyridin); $[\alpha]_D^{20} = +165^0$ ($\pm$ 3°) (c = 0,3 in Äthanol). Beim Trocknen für die Analyse im Hochvakuum bei 100° [15]) trat kein Gewichtsverlust ein.

$C_{16}H_{18}ON_2$	Ber. C 75,56	H 7,14	O 6,28	N 11,02	2 „H“ 0,79	1(C)CH_3 5,91%
(254,32)	Gef. „ 75,47	„ 7,27	„ 6,54	„ 11,21	„ 0,81	„ 6,44%

Potentiometrische Titration: 41,55 mg Substanz, gelöst in wässerigem Alkohol, verbrauchten 1,63 cm³ 0,1-n. HCl. Mol.-Gew. Ber. 254; Gef. 254. $pK_b = 7{,}45$.

UV.-Spektrum: Maxima bei 243 mμ (log $\varepsilon_{max} = 4{,}38$) und bei 313 m$\mu$ (log $\varepsilon_{max} = 4{,}04$) (vgl. Fig. 1). — *IR.-Spektrum:* siehe Fig. 2.

Farbreaktionen: Bei der *Keller*'schen und bei der *Van Urk*'schen Farbreaktion entsteht eine grüne Färbung, die sich nach gelbgrün verfärbt. Beim Auflösen einer Spur Setoclavin in konz. Schwefelsäure entsteht eine intensive, rein blaue Farbe, die mehrere Stunden beständig ist.

Hydrochlorid: Kristallisiert aus Wasser oder Alkohol in Nadeln, die sich ab 200° dunkel färben, ohne bis 300° zu schmelzen.

Nitrat: Aus Wasser Nadeln, die ab 125° dunkel werden, ohne bis 300° zu schmelzen. $[\alpha]_D^{20} = +190^0$ (c = 0,5 in 50-proz. Alkohol).

b) Isosetoclavin: Aus Methanol kristallösungsmittel-freie Polyeder. Smp. 234 bis 237°. Löst sich bei Siedehitze in 70 Teilen Methanol, 60 Teilen Aceton, 100 Teilen Essigester, 160 Teilen Chloroform und kristallisiert beim Erkalten aus allen diesen Lösungsmitteln. $[\alpha]_D^{20} = +107^0$ ($\pm$ 2°), $[\alpha]_{5461}^{20} = +147^0$ ($\pm$ 3°) (c = 0,5 in Pyridin); $[\alpha]_D^{20} = +129^0$ ($\pm$ 2°) (c = 0,4 in Äthanol).

$C_{16}H_{18}ON_2$	Ber. C 75,75	H 7,14	O 6,28	N 11,02	2 „H“ 0,79	1(C)CH_3 5,91%
	Gef. „ 75,65	„ 7,07	„ 6,52	„ 11,26	„ 0,79	„ 6,37%

Potentiometrische Titration: 36,2 mg Substanz, gelöst in wässerigem Alkohol, verbrauchten 1,41 cm³ 0,1-n. HCl. Mol.-Gew. Ber. 254; Gef. 256. $pK_b = 7{,}95$.

UV.-Spektrum: Maxima bei 242 mμ (log $\varepsilon_{max} = 4{,}42$) und bei 317 m$\mu$ (log $\varepsilon_{max} = 4{,}10$), siehe Fig. 1. — *IR.-Spektrum:* siehe Fig. 2.

Farbreaktionen: Wie Setoclavin.

Hydrochlorid: Kristallisiert aus Methanol beim Verdünnen mit Aceton in Rosetten. Ab 160° Dunkelfärbung, bis 300° noch nicht geschmolzen.

Pikrat: Aus Methanol orangegelbe Nadeln. Smp. 150—155°.

Die meisten Salze kristallisieren schlecht und zersetzen sich in Lösung sehr schnell.

c) Penniclavin. Ergänzung zu den früher mitgeteilten Daten[2]): $pK_b = 7{,}40$ (bestimmt durch potentiometrische Titration in wässerigem Alkohol mit 0,1-n. HCl).

Penniclavin-di-p-toluyl-L-tartrat: Kristallisiert aus Methanol, worin das Salz ziemlich schwerlöslich ist, in groben Rhomben. Smp. 173° (Zers.).

d) Isopenniclavin. Kristallisiert aus Wasser, in dem es bei Siedehitze zu 1% löslich ist, in sechseckigen Platten, ohne Kristallösungsmittel. In Methanol und Aceton

[15]) Alle Substanzen, bei denen nichts anderes erwähnt ist, wurden für die Analyse auf diese Weise getrocknet.

ist das Alkaloid sehr leicht, in Essigester leicht, in Chloroform mässig löslich. Smp. 163 bis 165°. $[\alpha]_D^{20} = +146°$ ($\pm$ 2°), $[\alpha]_{5401}^{20} = +198°$ ($\pm$ 3°) (c = 0,7 in Pyridin); $[\alpha]_D^{20} = +140°$ ($\pm$ 2°) (c = 0,9 in Äthanol).

$C_{16}H_{18}O_2N_2$	Ber.	C 71,09	H 6,71	O 11,84	N 10,36%	
(270,32)	Gef.	„ 71,22	„ 6,71	„ 12,15	„ 10,06%	

Potentiometrische Titration: 33,45 mg Substanz verbrauchten in wässerigem Alkohol 1,23 cm³ 0,1-n. HCl. Mol.-Gew. Ber. 270; Gef. 272. $pK_b = 8,10$.

UV.-Spektrum (siehe Fig. 1): Maxima bei 242 mμ (log $\varepsilon_{max} = 4,31$) und bei 313 mμ (log $\varepsilon_{max} = 3,94$). — *IR.-Spektrum:* siehe Fig. 2.

Farbreaktionen: Wie beim Setoclavin beschrieben.

Di-α-naphtoyl-D-bitartrat: Aus Methanol, worin das Salz schwerlöslich ist, in verfilzten Nadeln. Smp. 186° (Zers.).

e) Chanoclavin. Aus Aceton oder Methanol in dicken Prismen und Polyedern. Löst sich bei Siedehitze in 25 Teilen Methanol, 140 Teilen Aceton, 170 Teilen Essigester, 350 Teilen Chloroform. In Wasser sehr schwer löslich. Smp. 220—222°. $[\alpha]_D^{20} = -240°$ ($\pm$ 3°), $[\alpha]_{5401}^{20} = -294°$ ($\pm$ 4°) (c = 1,0 in Pyridin); $[\alpha]_D^{20} = -205°$ ($\pm$ 3°) (c = 0,75 in Alkohol).

$C_{18}H_{20}ON_2$	Ber.	C 74,96	H 7,87	O 6,24	N 10,93	1(C)CH_3	5,87%
(256,34)	Gef.	„ 75,24	„ 8,11	„ 6,34	„ 10,97	„	5,77%

Potentiometrische Titration: 42,1 mg Substanz verbrauchten in wässerigem Alkohol 1,65 cm³ 0,1-n. HCl Mol.-Gew. Ber. 256; Gef. 255. $pK_b = 5,80$.

UV.-Spektrum: Maxima bei 225 mμ (log $\varepsilon_{max} = 4,44$), bei 284 mμ (log $\varepsilon_{max} = 3,82$) und bei 293 mμ (log $\varepsilon_{max} = 3,76$), siehe Fig. 1. — *IR.-Spektrum:* siehe Fig. 3.

Farbreaktionen: Violettblaue Färbung bei der *Keller*'schen und bei der *Van Urk*'schen Farbreaktion, gleich wie Dihydro-lysergsäure, Elymoclavin und Agroclavin.

Bioxalat: Aus Wasser oder Methanol Nadeln. Smp. 195—197°. $[\alpha]_D^{20} = -152°$ (c = 0,5 in 50-proz. Alkohol).

O,N-Diacetyl-chanoclavin: 512 mg Chanoclavin wurden in 10 cm³ Pyridin und 2,0 cm³ Essigsäureanhydrid 24 Std. bei Raumtemp. stehengelassen. Die übliche Aufarbeitung lieferte 666 mg Diacetyl-Verbindung. Aus Benzol/Petroläther lanzettenförmige Nadeln. Smp. 174—175°. Die Kristalle halten 1 Mol Kristall-Benzol hartnäckig zurück, weshalb die Verbindung für die Analyse im Hochvakuum bei 180° sublimiert wurde. $[\alpha]_D^{20} = -55°$ (c = 0,9 in Pyridin.)

$C_{20}H_{24}O_3N_2$	Ber.	C 70,56	H 7,11	O 14,10	N 8,23%
(340,41)	Gef.	„ 70,19	„ 7,24	„ 14,19	„ 8,20%

IR.-Spektrum: Starke Banden bei 1630 cm^{-1} (Säureamid) und bei 1740 cm^{-1} (Ester), siehe Fig. 3.

Farbreaktionen: Gleich wie Chanoclavin.

Die Verbindung ist neutral; sie blieb beim Ausschütteln der ätherischen Lösung mit wässeriger Weinsäure in der organischen Phase.

N-Acetyl-chanoclavin: 340 mg Diacetyl-chanoclavin wurden in 7 cm³ 2-n. 50-proz. wässerig-alkoholischer KOH 2 Std. unter Rückfluss gekocht. Nach Verdünnen mit Wasser wurde das neutrale Verseifungsprodukt mit Chloroform/Isopropylalkohol extrahiert und aus Methanol umkristallisiert. Ausbeute 245 mg. Massive Prismen. Smp. 226—228°. $[\alpha]_D^{20} = -80°$ (c = 0,5 in Pyridin).

$C_{18}H_{22}O_2N_2$	Ber.	C 72,45	H 7,43	O 10,72	N 9,39	2 „H“	0,68%
(298,37)	Gef.	„ 72,59	„ 7,72	„ 10,94	„ 9,67	„	0,76%

Farbreaktionen: Wie Chanoclavin.

Verseifung von N-Acetyl-chanoclavin. 150 mg N-Acetyl-chanoclavin wurden in 5 cm³ 5-n. wässerig-alkoholischer KOH im Bombenrohr 2 Std. auf 170° erhitzt. Die dunkle Lösung extrahierte man mit Chloroform/Isopropylalkohol und chromatographierte den Rückstand an Aluminiumoxyd. Neben viel Zersetzungsprodukten wurden

28 mg einer Fraktion abgetrennt, die aus Aceton kristallisierte und 10 mg Chanoclavin lieferte. $[\alpha]_D^{20} = -240^0$ (Pyridin). IR.-Spektrum identisch mit dem eines authentischen Präparates.

4. Nachweis der Glykolgruppierung im Penniclavin und Isopenniclavin. a) 27 mg Penniclavin wurden in 3 cm³ 0,1-n. HJO_4 + 7 cm³ Wasser 1,5 Std. bei Raumtemp. stehengelassen. Dann destillierte man ca. 2/3 der Lösung in eine eisgekühlte Vorlage, versetzte das Destillat mit einigen Tropfen Natronlauge und 0,5 cm³ einer 10-proz. alkoholischen Dimedon-Lösung und erhitzte 10 Min. auf 80°. Das nach Abkühlen und Ansäuern mit Essigsäure ausfallende Kondensationsprodukt wurde aus Methanol/Wasser umkristallisiert. Ausbeute 13 mg (45% d. Th.); Smp. 187°, Misch-Smp. mit einem aus Formaldehyd und Dimedon hergestellten Vergleichspräparat ohne Depression.

$C_{17}H_{24}O_4$	Ber. C 69,83	H 8,28	O 21,89%
(292,36)	Gef. „ 69,66	„ 8,26	„ 21,79%

b) 27 mg Isopenniclavin lieferten unter den gleichen Bedingungen bei der Perjodat-Oxydation 18 mg (62%) des Formaldehyd-Dimedon-Kondensationsproduktes.

5. Oxydative Umwandlung von Elymoclavin in Penniclavin und Isopenniclavin. 6,5 g Elymoclavin wurden mit einem Äquivalent Schwefelsäure in 250 cm³ 50-proz. wässerigem Aceton gelöst. Zu dieser auf 70° erwärmten Lösung wurde in einem Guss eine gleich temperierte Lösung von 7,5 g $K_2Cr_2O_7$ in 250 cm³ Wasser gegeben. Nach 1 Min. setzte man 13 cm³ 2-n. H_2SO_4 zu und hielt den Ansatz noch 15 Min. bei 70°. Nach dem Erkalten wurde von einem unlöslichen Rückstand (9,0 g) abfiltriert und das Filtrat mit Chloroform/Isopropylalkohol 3:1 ausgezogen. Der Extraktrückstand (3,2 g) wurde an Aluminiumoxyd mit Chloroform und steigenden Zusätzen an Methanol chromatographiert. Mit Chloroform/3% Methanol liessen sich zwei Fraktionen eluieren. Die weniger gut haftende (0,2 g) gab mit Di-α-naphthoyl-D-weinsäure in geringer Ausbeute ein kristallisiertes Salz, das bei der Zerlegung 18 mg kristallisiertes, reines Isopenniclavin lieferte. Smp. 160—162°. $[\alpha]_D^{20} = +145^0$ (Pyridin).

Die besser haftende Fraktion (0,83 g) wurde über das Di-p-toluyl-L-tartrat gereinigt das aus Methanol gut kristallisierte. Aus diesem Salz liessen sich 0,52 g Penniclavin gewinnen. Smp. 215—225°. $[\alpha]_D^{20} = +151^0$ (Pyridin).

6. Oxydative Umwandlung von Agroclavin in Setoclavin und Isosetoclavin. 4,76 g Agroclavin wurden, gleich wie beim Elymoclavin beschrieben, in schwefelsaurer Lösung mit Dichromat oxydiert.

Aus der filtrierten Oxydationslösung liessen sich nach dem Alkalisieren mit $NaHCO_3$ mit Chloroform/Isopropylalkohol 3,8 g eines Alkaloidgemisches extrahieren, aus dem beim Aufnehmen mit Methanol 2,7 g Setoclavin auskristallisierten. Nach zweimaligem Umkristallisieren aus Aceton und Methanol wurden 2,0 g dieses Alkaloides mit den Daten der reinen Verbindung erhalten.

Aus dem Rückstand der Methanol-Mutterlauge des Setoclavins liessen sich durch Chromatographie an Aluminiumoxyd 0,65 g analysenreines Isosetoclavin (Smp. 234—237°, $[\alpha]_D^{20} = +106^0$ (Pyridin)) und weitere 0,38 g Setoclavin (Smp. 225—230°, $[\alpha]_D^{20} = +168^0$ (Pyridin)) gewinnen. Die Gesamtausbeute an krist. Setoclavin und Isosetoclavin beträgt 66% d. Th.

7. Reduktive Umwandlung von Chanoclavin in Festuclavin. Eine Lösung von 256 mg Chanoclavin (1 Millimol) und 128 mg Oxalsäure in 20 cm³ 90-proz. Alkohol wurde zu 150 mg, im gleichen Lösungsmittel vorhydrierten Pd-Mohr gegeben und bei Raumtemp. in der Schüttelente hydriert, bis 1 Millimol H_2 aufgenommen worden war.

Das durch die übliche Aufarbeitung gewonnene Reduktionsprodukt (230 mg) wurde an der 100fachen Menge Aluminiumoxyd chromatographiert, wodurch es sich in drei Fraktionen zerlegen liess. Die am raschesten wandernde Substanz, die mit abs. Chloroform eluiert wurde, kristallisierte aus Aceton in Nadeln, Ausbeute: 38 mg, Smp. 242—244°, $[\alpha]_D^{20} = -110^0$ (Pyridin). Sie war identisch mit Festuclavin[13]).

Eine Mittelfraktion, die mit Chloroform/0,5% Methanol eluiert wurde, war amorph und konnte nicht identifiziert werden. Mit Chloroform/2% Methanol wurde noch wenig unverändertes Chanoclavin herausgelöst.

Die Analysen wurden in unserem mikroanalytischen Laboratorium (Leitung Dr. *W. Schöniger*) ausgeführt. Die UV.- und IR.-Spektren wurden in unserer spektralanalytischen Abteilung (Leitung Dr. *H. G. Leemann*) aufgenommen.

SUMMARY.

By selection of certain strains of the ergot fungus found on *Pennisetum typhoideum Rich.* (a tropical millet) and modifying the substrate we succeeded in increasing the alkaloid yield in saprophytic cultures up to 1000—1500 mg alkaloids per liter of culture filtrate and increasing the alkaloid content of the mycelium to 1% of its dry weight.

From these *in vitro* cultures we succeeded in isolating four new ergot alkaloids besides the three previously obtained. The new alkaloids are *isopenniclavin* (formula VI), the isomer of penniclavin (formula V); *setoclavin* (formula III) and *isosetoclavin* (formula IV), an alkaloid pair; and *chanoclavin* (formula IX), a tricyclic secondary base. Chanoclavin is a new type of ergot alkaloid, containing an opened D ring. The complete structural formulas and configurations of the new alkaloids have been described.

Pharmazeutisch-Chemisches Laboratorium *Sandoz*, Basel.

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151. Untersuchungen über den sterischen Verlauf säurekatalysierter Cyclisationen bei terpenoiden Polyenverbindungen.

1. Mitteilung.

Cyclisation der 7,11-Dimethyl-2(trans),6(trans),10-dodecatrien- und der 7,11-Dimethyl-2(cis),6(trans),10-dodecatrien-säure

von **P. A. Stadler**[1]), **A. Nechvatal**[2]), **A. J. Frey** und **A. Eschenmoser.**

Herrn Prof. Dr. *T. Reichstein* zum 60. Geburtstag gewidmet.

(29. V. 57.)

A. Einleitung.

Die Fortschritte der Konstitutionsforschung auf dem Gebiete der alicyclischen Terpenverbindungen während der letzten Jahre und vor allem die überraschenden Ergebnisse der jüngsten experimentellen Forschung über die Biogenese von Vertretern dieser Naturstoffklasse haben nicht zuletzt auch ein erhöhtes Interesse an dem schon lange

[1]) Vgl. auch *P. Stadler*, Diss. ETH Zürich, 1957.

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TI Ergot alkaloid biosynthesis by isolates of Balansia epichloe and B. henningsiana
AU Bacon, Charles W.; Porter, James K.; Robbins, Joe D.
CS Richard B. Russell Res. Cent., USDA, Athens, GA, 30613, USA
SO Can. J. Bot. (1981), 59(12), 2534-8

TI Conformations of the ergot alkaloids chanoclavine-1, aurantioclavine, and N-acetylaurantioclavine [Claviceps purpurea, Fungi].
AU Sakharovskii, V.G.; Aripovskii, A.V.; Baru, M.B.; Kozlovskii, A.G.
AV DNAL (QD241.K453)
SO Chemistry of natural compounds., Sept/Oct 1983 (pub. 1984) Vol. 19, No. 5. p. 626-627

TI Peptide-type ergot alkaloids produced by Hypomyces aurantius
AU Yamatodani, Saburo; Yamamoto, Isao
CS Kobe Women's Junior Coll., Kobe, 650, Japan
SO Nippon Nogei Kagaku Kaishi (1983), 57(5), 453-6

Biosynthesis of ergot alkaloids. Mechanism of the conversion of chanoclavine-I into tetracyclic ergolines
AU Floss, Heinz G.; Tcheng-Lin, Marie; Chang, Ching-Jer; Naidoo, Bala; Blair, Garre E.; Abou-Chaar, Charles I.; Cassady, John M.
CS Dep. Med. Chem., Purdue Univ., West Lafayette, Indiana, USA
SO J. Amer. Chem. Soc. (1974), 96(6), 1898-909

TI Ergot alkaloid identification in clavicipitaceae systemic fungi of pasture grasses
AU Porter, James K.; Bacon, Charles W.; Robbins, Joe D.; Betowski, Don
CS Richard B. Russell Agric. Res. Cent., United States Dep. Agric., Athens, GA, USA
SO J. Agric. Food Chem. (1981), 29(3), 653-7

TI ALKALOIDS FROM THE FUNGUS CLAVICEPS-SP IBPM-F-401.
AU KOZLOVSKII A G; ARINBASAROV M U; SOLOV'EVA T F; ADANIN V M; GRIGOROV I; ANGELOV T I; SLOKOSKA L S; ANGELOVA M B
CS INST. BIOCHEM. PHYSIOL. MICROORG., ACAD. SCI. USSR, PUSHCHINO, USSR.
SO PRIKL BIOKHIM MIKROBIOL, (1980) 16 (4), 569-577.

90:199927
TI Ergosine, ergosinine, and chanoclavine I from Epichloe typhina
AU Porter, James K.; Bacon, Charles W.; Robbins, Joe D.
CS Richard B. Russell Agric. Res. Cent., Sci. Educ. Adm., Athens, Ga., USA
SO J. Agric. Food Chem. (1979), 27(3), 595-8

I LABORATORY PRODUCTION OF ERGOT ALKALOIDS BY SPECIES OF BALANSIA.
AU BACON C W; PORTER J K; ROBBINS J D
CS US FIELD CROPS LAB., US SCI. EDUC. ADM., R. B. RUSSELL AGRIC. RES. CENT., ATHENS, GA. 30604, USA.

which supports $R_1 = C_3H_7$ and $R_2 = C_4H_9$. This analogy may be applied to the remainder of the CI mass spectra for these alkaloids.

Since no CI study was made on the epimeric species of these compounds, it is unknown if the observed differences among the α- and β-ergosine, ergoptine, and ergokrystine (Table I) may be used to differentiate the alkyl moieties when R_2 = isobutyl and/or *sec*-butyl. Also, it is unknown at present if the differences in ion intensities for C′ (Table I) is a reflection of the stability and/or ease of formation of C relative to the substituents R_1 and R_2. However, the data indicate that CI mass spectrometry is an effective and useful complement to the EI spectra for the identification of the ergot peptide alkaloids.

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Ergot Alkaloid Identification in Clavicipitaceae Systemic Fungi of Pasture Grasses

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The isolation and identification of an alkaloid from *Balansia epichloë*, *Balansia strangulans*, and *Epichloë typhina* that corresponds to 6,7-secoagroclavine (UV; TLC; *m/e*) are reported. We report on the production of agroclavine, elymoclavine, penniclavine, and festuclavine by *E. typhina*. In addition, the two ergot peptide alkaloids from *E. typhina* previously listed as ergosine and ergosinine when analyzed with isobutane chemical ionization mass spectroscopy corresponded to ergovaline and ergovalinine. Another systemic fungus, *Balansia henningsiana*, was shown to produce chanoclavine(s), dihydroelymoclavine, and another presently unidentified ergoline alkaloid.

Previous investigations (Bacon et al., 1979; Porter et al., 1978, 1979a,b) of systemic fungi from toxic pasture grasses established that *Balansia epichloë*, *Balansia claviceps*, *Balansia henningsiana*, *Balansia strangulans*, and *Epichloë typhina* produced clavine-type alkaloids in vitro. Bacon et al. (1979) showed that several alkaloids produced by *B. epichloë* in vitro were also produced in vivo on parasitized smut grass (*Sporobolus poiretii*). These studies suggest that these systemic grass pathogens should be suspect in ergot toxicity syndromes of cattle and that "ergot" alkaloid biosynthesis occurs in other genera of Clavicipitaceae. The toxins responsible for the "ergot-like" syndromes observed in cattle have not been established. Therefore, it is important to characterize the alkaloids produced by these systemic grass pathogens.

Laboratory studies have shown similarities in the capability of *Balansia* and *Epichloë* to produce clavine al-

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kaloids. The major differences in alkaloid biosynthesis between *Balansia* and *Epichloë* is that *E. typhina* may be distinguished by its ability to produce ergot peptide alkaloids similar to *Claviceps purpurea* (Brunner et al., 1979). In addition to the alkaloids that chromatographed with ergosine and ergosinine (Porter et al., 1979a) and chanoclavine I, we also report the identification of agroclavine, elymoclavine, penniclavine, and 6,7-secoagroclavine from a 20-week culture of *E. typhina*. All of these clavine alkaloids correspond to authentic standards as described (Fehr, 1967; Porter et al., 1978, 1979b). The two ergot peptide fractions labeled ergosinine and ergosine (Porter et al., 1979a) also corresponded with synthetic standards except for the minor discrepancies (i.e., ion intensities) in the low-resolution mass spectra as reported. These, along with an absence of the molecular ion, the low abundance of the diagnostic fragments of *m/e* 280, 210, and 86 amu, and the close similarity of the spectra of the other homologous alkaloids (Brunner et al., 1979; Stadler et al., 1977), prompted further investigations into the identity of the ergot peptide alkaloids produced by *E. typhina*. Comparative TLC and low-resolution mass spectra eliminated all of the natural and synthetic alkaloids of the ergotoxine and ergoxine groups (Brunner et al., 1979) and all but ergosine and ergovaline from the ergotamine group. Interpretation of ergovaline's mass spectra (i.e., electron impact, EI, at 70 eV) is complicated by the fragmentation of the tricyclic peptide moiety resulting in the same atomic mass ions as the lysergic acid amide and clavine portions (Vokoun et al., 1974; Vokoun and Rehacek, 1975) of the alkaloid. The above is further compounded by the almost identical chemical and physical properties of ergosine and ergovaline (Brunner et al., 1979) and the problems of working with small quantities of a natural isolate (Porter et al., 1979a). Chemical ionization (CI) mass spectroscopy's (Arsenault, 1972; Falco et al., 1970) compliment to the low-resolution electron impact (EI) has been reported for differentiation of the alkyl substituents attached to the tricyclic peptide moiety of these alkaloids (Porter and Betwoski, 1981). Therefore, CI mass spectroscopy was used to compare the natural peptide alkaloids from *E. typhina* with authentic ergosine and ergovaline.

Cultures of *B. epichloë* and *B. strangulans* were reported (Bacon et al., 1979; Porter et al., 1979a,b) to produce a clavine alkaloid (M^+ 240) that we now report as 6,7-secoagrocalvine. The identification was based on the comparison with a synthetic standard using ultraviolet (UV) and mass spectroscopy, cochromatography, and color reaction (blue) with *p*-(dimethylamino)benzaldehyde (PDAB) and also by a comparison of the methylated derivative of the natural alkaloids with synthetic *N*-methyl-6,7-secoagroclavine (Fehr, 1967). The 6,7-secoagroclavine has been recently reported as a natural product from *C. purpurea* (Horwell and Verge, 1979).

EXPERIMENTAL SECTION

Organisms, culture, and alkaloid extraction, isolation, and detection methods were as previously described: *B. epichloë* (RRC 242), alkaloid no. 1 (Porter et al., 1978, 1979b); *B. strangulans* (RRC 233), alkaloid fraction (Bacon et al., 1979) and alkaloid *m/e* 240 (Porter et al., 1978); *E. typhina* (RRC 238), ergot peptide alkaloids labeled ergosine and ergosinine (Porter et al., 1979a); *E. typhina* (RRC 238), a 20-week culture on media 104 (Porter et al., 1979a). In addition, *E. typhina* (RRC 238) was cultured for 12 weeks on 100 g of sorbitol, 10 g of glutamic acid, 1.0 g of yeast extract, 1.0 g of KH_2PO_4, 0.3 g of $MgSO_4 \cdot 7H_2O$, and 1000 mL of distilled water, and the pH adjusted to 5.6 with NH_4OH, and the *B. henningsiana* (RRC 243) alkaloid fraction obtained from Bacon et al. (1979).

Chromatography. Thin-layer chromatography was performed on silica gel GF-254 according to reported procedures (Agurell, 1965; Porter et al., 1979a; Stahl, 1969) with the following solvent systems: chloroform–methanol (CM), 4:1 (v/v); chloroform–dimethylamine (CDEA), 9:1 (v/v) (Agurell, 1965); benzene–dimethylformamide (BDMF), 86.5:13.5 (v/v) (Stahl, 1969); chloroform–methanol–concentrated ammonia (CMA), 94:5:1 (Horwell and Verge, 1979); chloroform–methanol, 9:1 (v/v) in a saturated ammonia atmosphere (CMAtm) (Cassady et al., 1973); methylene chloride–2-propanol (MP), 3:1 (v/v).

Instrumental. Low-resolution electron impact mass spectra were obtained as reported (Porter et al., 1979a,b). Chemical ionization mass spectra were obtained via a direct inlet probe on a Varian Mat 44 quadrapole mass spectrometer equipped with an EI/CI source (~200 V). The ion source was maintained at a pressure of ~200 μbar and operated at a temperature of ~245 °C. The probe temperature was programmed at 20 °C/min from ambient to 250 °C. The fragment ions were observed between 175–200 °C. Only those ions occurring at a relative intensity greater than 1% are recorded. Isobutane was used as the reactant gas, and (perfluorotributyl)amine (*m/e* 219, 264, and 414 amu) was used as a calibration compound. Ultraviolet spectra were performed in methanol by using a Varian Cary 15 ultraviolet spectrometer. Infrared spectra were performed using KBr micropellets in a Perkin-Elmer Model 457A infrared spectrometer. Nuclear magnetic resonance spectra were performed in CD_3OD by using a JEOL PS/PFT 100. Melting points were uncorrected and obtained with a Mettler FP5 apparatus connected to a Mettler FP52 microfurnace.

Synthesis of 6,7-Secoagroclavine. *N*-Methyl-6,7-secoagroclavine [M^+ 254; mp 132–134 °C [lit. mp 136 °C (Fehr, 1967)]] was synthesized from agroclavine (Eli-Lilly) according to Bhattacharji et al. (1962) and separated from its Δ^7 isomer [M^+ 254; mp 120 °C [lit. mp 118–120 °C (Fehr, 1967)] by preparative TLC on silica gel GF 254 by using the CM solvent system (R_f 0.57 and 0.50, respectively). The *N*-methyl-6,7-secoagroclavine (0.4 mM) in 2 mL of anhydrous acetone was treated with 0.7 mM of diethyl azodicarboxylate (Aldrich Chemical Co.) in 3 mL of anhydrous diethyl ether. The reaction mixture was stirred under N_2 for 5 h and allowed to stand in the refrigerator overnight. The reaction mixture was extracted (3 × 3 mL) with a 2% tartaric acid solution. Extracts were combined, the pH was adjusted to ~10 with NH_4OH, and the resulting solution was extracted with 3 times the equivalent volume of $CHCl_3$. The alkaloidal extracts were combined, treated with anhydrous Na_2SO_4, filtered, and subjected to preparative TLC (20 × 20 cm glass plates, 0.75-mm silica gel GF-254) using the CDEA solvent system. The *N*-methyl-6,7-secoagroclavine (R_f 0.70; M^+ 254) starting material separated from the reaction products (R_f 0.60) in this system. Low-resolution mass analyses of the reaction product(s) isolated from the silica gel suggested it consisted of at least three compounds: M^+ 252, *m/e* 237 (252 – CH_3); M^+ 240, *m/e* 225 (240 – CH_3); M^+ 176, *m/e* 131 (176 – OC_2H_5). The desired product (M^+ 240) was isolated from this mixture by preparative TLC using the BDFA solvent system (R_f 0.37). After elution from the silica gel, this fraction was chromatographed in the CM solvent system (R_f 0.23), followed by recrystallization from hexane [mp 132 °C [lit. mp 138 °C (Fehr, 1967); lit. mp 126–129 °C (Horwell and Verge, 1979)]], and the isolated compound had a UV, IR, and NMR as reported (Fehr,

1967). Also isolated in this chromatography system was a fraction (R_f 0.57) that was visually observed as a dark blue band under 254 and 366 nm and gave a blue reaction to PDAB. Its absorption ($\lambda_{max}^{CH_3OH}$ 317 and 236 nm) along with the low-resolution mass spectrum (M^+ 252, m/e 237, 221, 206, 196, 181, 167, 154, and 85) gave indications that this fraction consisted mainly of a dehydrogenated *N*-methyl-6,7-secoagroclavine with the newly formed double bond being conjugated with the indole nucleus (Cassady et al., 1973). The mass spectrum also revealed the presence of diethyl hydrazinodicarboxylate (Fieser and Fieser, 1967; Yoneda et al., 1966), M^+ 176, m/e 131 and 130 amu, as a minor constituent.

Methylation of Natural Products (M^+ 240) Isolated from *B. epichloë* and *B. strangulans*. Methanol, 200 μL, and 10 drops of methyl iodide (Aldrich Chemical Co.) were added to sufficient chromatography material (~250 μg) of each natural alkaloid. The systems were flushed with N_2, capped, and allowed to stand for 45 min at 40–45 °C with periodic manual agitation. The reaction mixtures were concentrated under a stream of N_2 to 100 μL, and each was subjected to preparative TLC in CDEA as above. This system separated starting material from the semisynthetic product (M^+ 254), indicative of the addition of one methyl substitutent.

Festuclavine from *E. typhina* (Sorbitol–Glutamic Acid Culture Media). The alkaloid fraction (Porter et al., 1979a) from a 12-week culture was chromatographed on silica gel by using CM and was visualized by spraying with PDAB. The one band corresponding to a blue reaction for ergot alkaloids was scrapped from the TLC plate, eluted from the silica with chloroform–methanol, 1:1 (v/v), and chromatographed with authentic pyro-, costa-, and festuclavine in solvent systems CDEA and BDMF. The natural alkaloid from this culture corresponded to festuclavine as determined by UV, TLC, and m/e (Agurell, 1965; Vokoun et al., 1974).

Clavine Alkaloids from *B. henningsiana*. The alkaloid fraction (625 μg, based on ergonovine maleate) isolated from a 5-week culture of *B. henningsiana* (Bacon et al., 1979) was subjected to preparative TLC in solvent system CM and resulted in three fractions (R_f 0.10, 0.18, and 0.65) that gave a blue reaction with PDAB. Although fraction 1 (R_f 0.10) corresponded to chanoclavine I in CDEA (Agurell, 1965) and CMAtm (Cassady et al., 1973; Porter et al., 1978) and its low-resolution mass spectrum showed M^+ 256, m/e 237, 183, and 154 as reported (Vokoun et al., 1974), the quantity of material isolated was not sufficient to unequivocally eliminate the isomeric relatives. Fraction 2 (R_f 0.18) cochromatographed with authentic dihydroelymoclavine (dihydrolysergol I, Eli Lilly) in CM (R_f 0.18), CDEA (R_f 0.15), CMAtm (R_f 0.47), and MP (R_f 0.04). Comparison of the low-resolution mass spectra of this fraction and dihydroelymoclavine (Table I) conducted under identical conditions showed discrepancies which may be the results of compound purity or an isomer that will not separate by chromatography under the conditions described. Attempts to compare the acetylation material of fraction 2 with *O*-acetyldihydroelymoclavine synthesized according to Agurell et al. (1963) were unsuccessful, probably because of the quantity of the compound originally isolated. Fraction 3 (CM, R_f 0.65) had a $\lambda_{max}^{CH_3OH}$ and low-resolution mass spectrum (Figure 1) suggestive of either an isomer of dihydrolyseramide [CM, R_f 0.26 [lit. R_f 0.23 (Agurell, 1965)]] or a derivative of this compound, i.e., RCONHR′ for R = ergoline [cf. m/e 144, Figure 1 (Barber et al., 1965; Schmidt et al., 1978)] with the R′ unknown. Fermentation conditions for *B. henningsiana* and the other systemic fungi mentioned above are under investigation in order to provide material sufficient for absolute identification of these alkaloids.

Table I. Mass Fragmentation Comparison of Dihydroelymoclavine and Alkaloid Fraction 2 from *B. henningsiana* (cf. the Text)

	%	
m/e	standard	natural product
257 (M + 1)	15	15
256 (M)	68	55
241	5	5
239	2	7
237	3	7
225	8	13
223	16	15
211	2	15
209	6	13
207	5	16
197	24	25
195	7	10
194	9	16
182	15	22
168	26	27
167	35	39
159	1	16
156	14	17
155	31	36
154	100	100
153	13	22
144	39	39
127	36	45
115	13	21

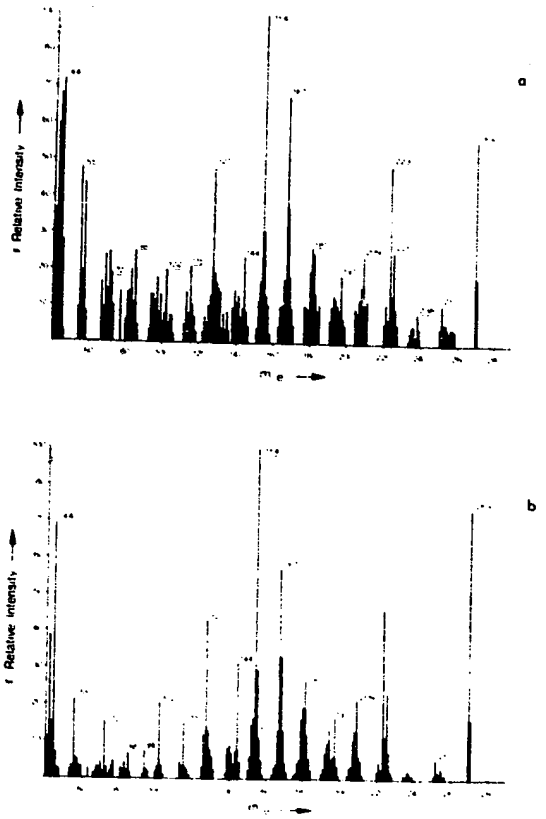

Figure 1. (a) Alkaloid (fraction 3) from *B. henningsiana* (cf. the text); (b) dihydrolyseramide.

Table II. Major Diagnostic Mass Fragments of Synthetic (syn.) 6,7-Secoagroclavine (Fehr, 1967) and Alkaloid(s) Isolated from Systemic Fungi *B. epichloë* (B.e.), *B. strangulans* (B.s.), and *E. typhina* (E.t.)

	%			
m/e	syn.	B.e.	B.s.	E.t.
240	100	100	100	100
225	10	12	10	17
208		12	12	17
197	16	15	18	37
194		20	21	27
184	22	25	28	32
182		23	26	46
168	37	42	45	54
155	59	58	79	82
127	10	18	26	37
115		18	20	15
85	56	75	79	85
42	17			29

RESULTS AND DISCUSSION

Alkaloid Workup of 20-Week Culture of *E. typhina* (RRC 238) on M 104. Preparative chromatography of the alkaloid fraction from Porter et al. (1979a) on silica gel GF 254 using CM resulted in six fractions that cochromatographed (R_f) with (1) ergosinine (0.74), (2) ergosine (0.60), (3) agroclavine (0.35), (4) penniclavine (0.26), (5) elymoclavine (0.21), and (6) chanoclavine(s) (0.09). After elution from the silica gel, these individual fractions chromatographed as homogeneous spots in the CM system with the authentic standards listed. Three of these fractions separated into additional compounds on chromatography in the CDEA solvent system. Fraction 1 separated into the alkaloid that cochromatographed with ergosinine and a minor compound that was visualized as a blue spot only after spraying with PDAB. This minor compound (R_f 0.76) was separated from ergosinine by developing the plates 2 times in CDEA and allowing the plates to air dry between runs. Although this minor component appeared homogeneous in CM (R_f 0.74), CDEA (R_f 0.54), and BDMF (R_f 0.79) and its UV spectrum suggested it was a simple indole or clavine alkaloid ($\lambda_{max}^{CH_3OH}$ 292, 281, 273, and 224 nm), the low-resolution mass analyses of this fraction suggested a complex mixture of several compounds. As the probe temperature of the mass spectrometer was increased in 50 °C increments from ambient to 250 °C, major ions were observed at 100 °C (*m/e* 244, 153, 125, 91, and 70 amu) and 250 °C (*m/e* 369, 314, 297, 270, 255, 237, 225, 207, 194, 192, 181, 168, 167, 154, 143, and 127 amu), respectively. The above spectra may represent two compounds, one of which may be (or contain) the phenylalanylproline lactam moiety (Groger et al., 1975); also, the observed spectra may represent one compound that is subject to pyrolyses prior to electron impact similar to the ergot cyclol alkaloids (Porter and Betowski, 1981). This fraction was stored under N_2 (0 °C) for future investigations.

When subjected to preparative chromatography in the CDEA system, fraction 5 separated into elymoclavine (Porter et al., 1979b) and another compound that corresponded [UV; TLC; *m/e* (Table II)] to the synthetic 6,7-secoagroclavine. Fraction 6 in the CDEA system consisted mainly of chanoclavine I (UV; TLC; *m/e*) and a few minor compounds that were not investigated.

The natural alkaloids [M^+ 240 (Table II)] from *B. epichloë* (Porter et al., 1978, 1979b), *B. strangulans* (Bacon et al., 1979; Porter et al., 1978), and *E. typhina* had identical chromatography behavior with synthetic 6,7-secoagroclavine in the CM (R_f 0.21), CDEA (R_f 0.57), and CMA systems with R_f values relative to those of elymoclavine and agroclavine (Horwell and Verge, 1979).

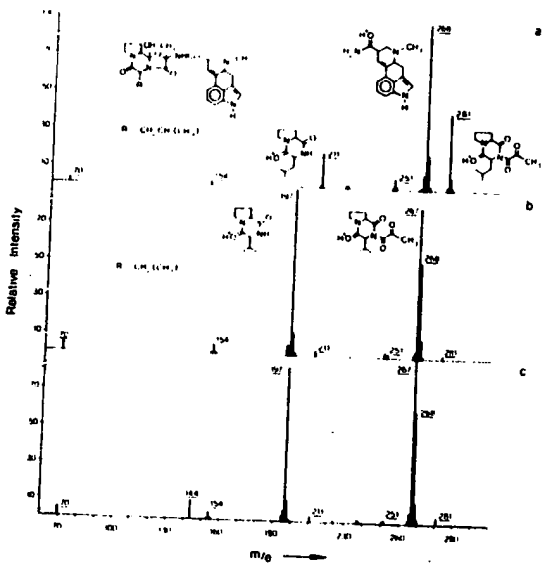

Figure 2. Isobutane chemical ionization mass spectra: (a) ergosine; (b) ergovaline; (c) peptide alkaloid (fraction 1) from *E. typhina* (cf. the text).

Although the natural product could not be separated from elymoclavine in CM and agroclavine in CDEA (Porter et al., 1979b), these two systems along with CMA (Horwell and Verge, 1979) may be used effectively for the preparative TLC isolation of this compound.

The low-resolution mass analyses for the above alkaloids are compared with that of 6,7-secoagroclavine (Fehr, 1967) in Table II. Also, the methylation product(s) of the above compounds corresponded with synthetic *N*-methyl-6,7-secoagroclavine on cochromatography in CM and CDEA as described (cf. synthesis).

The above data strongly suggest that the natural compounds isolated from *B. epichloë*, *B. strangulans*, and *E. typhina* are 6,7-secoagroclavine; however, paucity of the isolated materials prevented unequivocal establishment of conformation and therefore does not rule out the possibility of C-5, C-10(H) epimeric species.

Isobutane Chemical Ionization Mass Spectrometry of the Peptide Alkaloids from *E. typhina*. The electron impact (70 eV) mass spectrum of ergosine has been reported (Vokoun et al., 1974; Vokoun and Rehacek, 1975). The chemical ionization mass spectrum of ergosine is compared with that of ergovaline and the natural peptide alkaloid isolated from *E. typhina* in Figure 2. Under CI, the peptide fragment for ergosine corresponding to *m/e* 280 (Vokoun and Rehacek, 1975) undergoes the ion-molecule reaction with isobutane, thereby producing the abundant fragment at *m/e* 281 [42% (Figure 2a)]. Alternatively, ergosine may decompose via the diketopiperazine pathway (Porter and Betowski, 1981; Vokoun et al., 1974; Vokoun and Rehacke, 1975) which then undergoes the same reaction with isobutane, thus yielding *m/e* 211 (18%) (EI, *m/e* 210) and supporting R = C_4H_9. The lysergic acid amide fragment corresponding to *m/e* 267 undergoes the same mechanism, thereby resulting in *m/e* 268 (100%) for ergosine (Figure 2a). Similarly, the natural product from *E. typhina* (Figure 2c) resulted in *m/e* 267 (100%) and *m/e* 197 (95%), the peptide fragments indicative of R = C_3H_7 (Porter and Betowski, 1981). These two fragments corresponded to EI *m/e* 266 and 196, respectively (Porter et al., 1979a), and thus strongly support the identification of the natural compound from *E.*

typhina as ergovaline. The above ions for the natural product (Figure 2c) are also supported by the abundant lysergic acid amide fragment at *m/e* 268 (61%). The CI spectrum of natural ergovaline (i.e., from *C. purpurea*) is compared with the spectrum of the peptide alkaloid isolated from *E. typhina* (parts b and c of Figure 2, respectively). The minor differences observed in the relative intensities of *m/e* 267 and 197 between the two compounds may be a reflection of both instrument temperature and pressure at which pyrolysis and subsequent fragmentation occurs (Porter and Betowski, 1981) and possibly concentration of epimeric species. Figure 2b is the CI spectrum of ergovaline, whereas Figure 2c represents the spectrum of the epimeric mixture (i.e., ergovaline–ergovalinine) as determined by TLC.

The above natural standard (Figure 2b) was isolated from a fraction containing ergosine (Brunner et al., 1979), and thus it is unknown at present if the minor fragments occurring at *m/e* 281 and 211 in both spectra (Figure 2b,c) represents trace amounts of ergosine (Figure 2a). Pure synthetic ergovaline was unavailable for these comparisons. Thus, the possibility exists that *E. typhina* produces ergosine and ergosinine (Porter et al., 1979a) in trace amounts with ergovaline and ergovalinine as the major components.

The above comparisons do not eliminate the possibility of the peptide alkaloids from *E. typhina* as being isomeric relatives of ergovaline and ergovalinine. Conformational analyses of these alkaloids, as with the clavine alkaloid from *Balansia*, are predicated on quantities sufficient for these determinations.

Although the synthetic peptide alkaloids ergovaline and ergovalinine have been known for some time (Stadler et al., 1964), only recently have these alkaloids been reported as natural products (i.e., from cultures of *C. purpurea*) (Brunner et al., 1979).

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TI Alkaloids from the fungus Penicillium aurantio-virens Biourge and some aspects of their formation
AU Solov'eva, T. F.; Kuvichkina, T. N.; Baskunov, B. P.; Kozlovskii, A. G.
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CS Inst. Biochem. Physiol. Microorg., Pushchino, USSR
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SO AGRON J, (1986) 78 (1), 106-116.

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AU Abe, Matazo; Ohmomo, Sadahiro; Ohashi, Tsutomu; Tabuchi, Takeshi
CS Tokyo Univ. Educ., Tokyo, Japan
SO Nippon Nogei Kagaku Kaishi (1969), 43(8), 575-82
CODEN: NNKKAA
DT Journal

Ergot alkaloids from plants

to support the conclusion that the barrier action of the stubble was a significant mechanism.

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Ergot Toxicity from Endophyte-Infected Grasses: A Review[1]

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ABSTRACT

Clarifying the role of grasses parasitized by a tribe of clavicipitaceous endophytes (Balansiae) in the etiology of the many cattle (*Bos taurus*) ergot toxicity syndromes is essential for identifying the correct toxic grass and establishing the proper pasture management practice necessary to eliminate toxicity. The distinction is required as the management practice used to prevent ergot toxicity by *Claviceps* does not apply because of the systemic habit of the Balansiae. Research is reviewed, which established that endophytic parasites of many genera of warm-season perennial weed grasses and tall fescue (*Festuca arundinacea* Schreb.) are producers of toxic ergot alkaloids. This group of fungi is distinct from the closely related genus *Claviceps* in being an intercellular parasite of leaf tissue. These fungi are parasitic on nine tribes of grasses that have a wide geographic distribution in the western hemisphere. Nine ergot alkaloids belonging to the clavine group have been isolated from four species of *Balansia* cultured in laboratory media, and several of these have been isolated from parasitized grasses. Ergot alkaloid production by the remaining nine species of *Balansia* has not been examined. The endophyte of tall fescue produced both the clavine and ergotamine peptide groups of alkaloids in culture and in the grass. The production of ergot alkaloids by weed grass endophytes is host-related; therefore, each parasitized weed grass must be assessed for alkaloid production. All isolates of *B. epichloe* (Weese) Diehl from smutgrass (*Sporobolus poiretii* Roem. and Schult.) and 54% of the isolates of *B. henningsiana* (Moell.) Diehl from broomsedge (*Andropogon virginicus* L.) produced the following alkaloids in culture: chanoclavine, ergonovine, ergonovinine, and agroclavine. Isolates of these two fungi from other grass genera did not produce alkaloids. While no extensive survey has been done to date, all infected tall fescue examined contains ergot alkaloids. The main peptide alkaloid produced by the tall fescue endophyte is ergovaline. The effects of this peptide alkaloid on cattle have not been determined. However, the total alkaloids produced in culture by *B. epichloe* reduced the serum prolactin levels in cattle and along with prior published accounts on the physiological effects of the clavine and peptide ergot alkaloids, established that these alkaloids can cause toxicity symptoms in cattle consuming infected plant material. Thus, pasture management practices must include procedures that will prevent the growth of grasses that serve as host for endophytic fungi.

Additional index words: **Endophytic fungus, Fescue endophyte, Pasture toxic fungi, Cattle ergot toxicity, Clavicipitaceous systemic fungi, Parastic fungi of grasses, Toxic weed and fescue grasses.**

PASTURE systems are based on cattle (*Bos taurus*) performance, which results from nutrient intake of a specific forage or combination of grass and legume species. Theoretically ideal systems are unrealistic because the environment of any given pasture consists of dynamic ecological factors that create marked imbalances in the botanical composition. Although certain management strategies will help maintain a forage species in a pasture, there are a number of sites in most pastures that are unsuited for the intended species. Invariably, such areas serve as portals for a variety of forage or weed species. Many of these invading species may be grasses and have some redeeming forage value, but since they are not the grass species planted they are considered weeds. Once established, these weed grasses can compete with the forage species for more desirable sites in pastures, and, depending on the rigor of the management practice, occupy large acreages in time. According to the grazing habit of the animal, these weed grass species can have redeeming forage value and contribute significantly to the diet. Herein lies the problem. These weed grasses can be readily consumed by animals, but their contribution

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in the dietary intake of the animal is oftentimes overlooked. A performance problem that might be related to ingesting weeds may be attributed to the major pasture grass without regard to the total diet of the animal.

Toxicity in pastures is often attributed to toxic broadleaf weeds; however, in many cases the problem may be grass toxicity. Pasture grass toxicities are historically attributed to the major forage grass established in the pasture. Thus, there are the tall fescue (*Festuca arundinacea* Schreb.) toxicity syndrome (54), bermudagrass [*Cynodon dactylon* (L.) Pers.] tremors (12), dallisgrass (*Paspalum dilatatum* Poir.) poisoning (28), and ryegrass (*Lolium perenne* L.) staggers (23). It has been clearly demonstrated that these major grasses all have toxic constituents and at times pose problems to livestock. Interestingly, all of these toxic syndromes are similar to signs of ergot alkaloid poisoning resulting from ingesting sclerotia of *Claviceps* species and may be categorized as either gangrenous or convulsive ergotism. Examination of fescue pastures for *Claviceps* indicated that this fungus was absent; therefore, cattle toxicity was not produced by ingesting ergot sclerotia (54). Recently a systemic endophytic fungus, *Epichloe typhina* (Pers. ex Fr.) Tul. (*Acremonium coenophialum* Morgan-Jones and Gams), that is related to *Claviceps*, was isolated from cultivars of toxic tall fescue (6), and associated with poor cattle performance (42). Ryegrass toxicity is due to an endophytic fungus morphologically identical to that in fescue (23). Several grass species are parasitized by systemic fungi (11, 18, 24, 47), which suggests that these associations should be examined for similar toxicity problems in cattle.

We now have evidence that ergot-related pasture grass toxicity is not necessarily due to ingesting a major forage grass, but also to weed grasses parasitized by other endophytic fungi, species of *Balansia* and *Myriogenospora*, growing in association with the major pasture species. It is possible to have cattle show signs identical to those of fescue toxicity in a bahiagrass pasture where tall fescue is entirely absent. Thus, major pasture grasses might be blamed for poor cattle performance, when in fact toxicity is due to weed grasses. The purpose of this review is to present a series of studies that (i) describe the nature of the relationship between the endophytic parasite and its grass host; (ii) discuss the role played by a major forage grass, tall fescue, in cattle toxicities; (iii) clarify and indicate the role played by endophyte-weed associations in other cattle toxicity syndromes; and (iv) present recommendations concerning the use of endophyte-free fescue and weed grasses in pastures.

THE GRASS-FUNGUS ASSOCIATION

Weed Grasses

These endophytes are parasitic fungi belonging to a small obscure tribe (Balansiae) of the Clavicipitaceae family, and are distinguished from the infamous and closely related genus *Claviceps* by their endophytic habit. Species of *Claviceps* are strictly localized ovarian parasites whose hyphae only penetrate the vascular system of the host floret (25). All the species of *Balansia*, as well as other members of the Balansiae (*Atkinsonella* and *Epichloe*) are completely systemic, hyphae are found between cells of various tissues of the leaf and inflorescence stem (Fig. 1a). *Myriogenospora atramentosa* (Berk. et Curt apud Berk) Diehl is the only exception to the endophytic habit in being localized externally (superficially systemic) on the surface of leaves and developing flowers (24). Once a grass is parasitized by an endophyte species, it remains infected. While all *Balansia* species have the systemic habit in common, there are marked morphological differences between species. Based on these morphological variations, Diehl (11) recognized 13 species in the western hemisphere with four additional species having been reported from various locations in India (43). The endophytic hypha of each species appears similar within the tissue and cannot be distinguished. A species of *Balansia* is distinguished by the location of the stromata (fruiting bodies) on the grass. For example, *B. epichloe* (Weese) Diehl produces its stroma on the upper surface of the host leaf, whereas *B. strangulans* (Mont.) Diehl produces its stroma at the nodes of its host (Fig. 1b, 1e). Detailed morphological studies have not been done on each species; however, we feel that each species cannot be distinguished on the basis of the morphology of the asexual stroma (39). The fine systemic hyphae are intercellular, run parallel with the long axis of the host cells, and occasionally there are branched hyphae that run horizontally for a short distance (Fig. 1a). At areas of exits from the plant (node, leaf surface, etc.) that are peculiar to the fungal species, the very fine systemic hyphae become coarse and wide as they form the external stromata. The hyphae may exit by either separating two epidermal cells or grow through the stomata (38).

Evidence of infection in grasses by species of *Balansia* is not obvious without the appearance of external stromata. The usual necrotic lesions and other obvious symptoms that are characteristic of fungal parasitized plants are not produced by these fungi. The first sign of infection is the appearance of varying shades of white to black stromata on the leaves on flowering stem and parts of infected grasses (Fig. 1b–1g). The stromata are structured within which spores are produced and are useful indicators of infection. The role of these spores in infection is based largely on the observation that formation of the stromata occurs on the outside of the grass at the time noninfected host species are flowering and on the assumption that infection takes place through the flower. Since any one pasture would contain several weed grasses, each pasture must be monitored throughout its flowering periods (spring, summer, or fall) to macroscopically determine infection of each weed species. It is expected that the spores produced on the stromata infect the ovule through the stigma; therefore, the parasitism is seed-borne, similar to the endophyte of tall fescue (3, 39, 47). But unlike the endophyte of tall fescue, the species of *Balansia* produce external spores, which suggest that they have the potential to infect healthy plants. Infection taking place through the ovule is a generalization for all species and is based on the meager but successful attempts by Diehl (11), who infected a grass with one species of *Balansia*. In this laboratory numerous attempts at infection have failed. We have

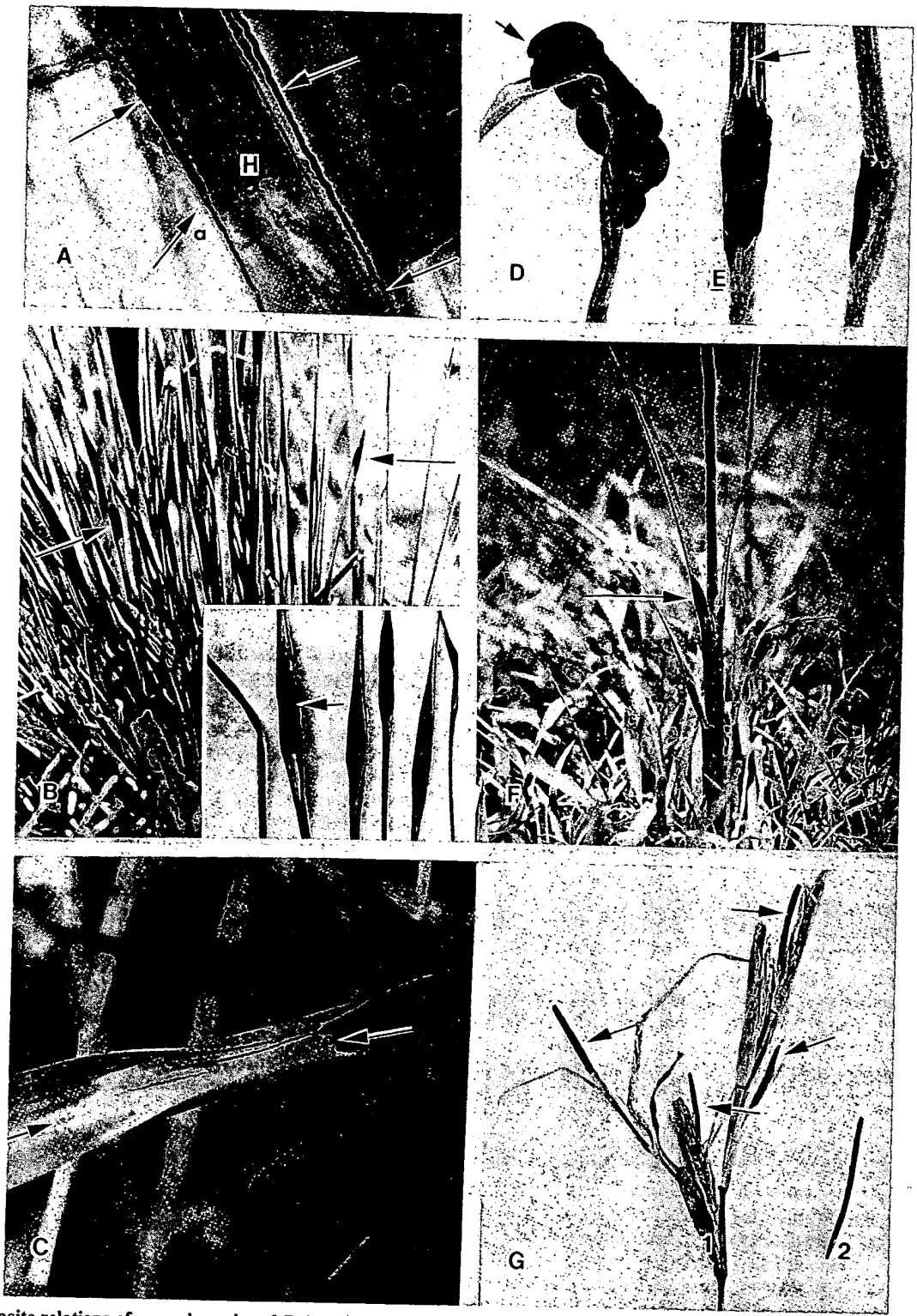

Fig. 1. Host-parasite relations of several species of *Balansia*. *A:* Endophytic mycelium of *B. strangulana* (arrows) located between cells of pith tissue from *Panicum hians*, H; arrow at *a* shows a branched hypha. The appearance of other species of endophytes are similar. *B: B. epichloe* on *Sporobolus poiretii* (smutgrass), (arrows); insert shows a close-up of the black ascomata (arrow) on the upper surface of the leaf and within which ascospores are produced late in the flowering season. *C: B. epichloe* on smutgrass showing the white to gray conidiomata between the arrows that is produced early in the flowering season of the year. *D: B. pilulaeformis* on an aborted inflorescence upon which the black ascomata is produced (arrow) extending over onto the flag leaf. *E:* Ascomata of *B. strangulans* on the nodes of two stems of *Panicum hians*; note that the stroma on the left (arrow) still has some pale white conidiomata remaining. *F:* Ascomata (arrow) of *B. henningsiana* produced on lower leaf blade of *Panicum tenerum*. *G:* Several ascomata, arrows of *B. obtecta* and one ascomatum (G2) removed from a floret of *Cenchrus echinatus*. To demonstrate this fungus, the outer leaves were removed from several aborted florets; note the striking similarity of this species to the sclerotia of *Claviceps*.

observed on several occasions the development of an infected plant from seed but this was not experimentally produced. Infection is maintained within a plant because of the perennial nature of the systemic hyphae associated with meristematic tissues of the host.

The information on the mode of infection and spread of the fungus stated above is concerned with chasmogamous flowers and seed. However, certain grass species are characterized by the production of two types of flowers, chasmogamous, and cleistogamous. A recent study indicated that *Danthonia* normally produces both types, but when infected with the endophyte *Atkinsonella* it produced only cleistogamous flower that set seeds, which were capable of germinating and transmitting the *Atkinsonella* infection (9). The infection of cleistogamous flowers and dissemination of cleistogamous seeds by *Balansia*-infected grasses have not been examined but merit attention. Nevertheless, the species of the Balansiae are considered to follow the generalized life cycle of other endophytic fungi (Fig. 2).

Tall Fescue

The production of external fructification is by no means the rule as symptomless systemic fungal infections occur in perennial ryegrass (*Lolium perenne* L.) (23) and several species of *Festuca* L. (41, 50). The symptomless infection suggests that this relationship is extremely compatible. However, it poses a problem relative to fungus identification. On laboratory media, the endophyte of tall fescue produces only the asexual state: slimy, hyaline, ellipsoidal conidia borne on phialids. This asexual state was assigned the name *Sphacelia typhina* by Saccardo in 1881 (40), and it was not until 1887 when this asexual state was established as being connected with the sexual state of *Epichloe typhina* (Pers. ex Fr.) Tul (10), the choke disease of grasses. The endophyte of fescue was initially referred to as *S. typhina* (6, 41). Recently, Morgan-Jones and Gams (29) on the basis of morphology of media-cultured fungi transferred the asexual state of the choke disease from *Sphacelia* to *Acremonium typhinum* and considered the fungus in tall fescue to differ significantly to warrant a new species, *A coenophialum*.

Figure 2 presents the life cycle of the tall fescue endophyte as it is presently defined. The endophyte is a seed-borne parasite in which the fungus hyphae are deeply sequestered between the epidermal cells of the scutellum and the starchy endosperm. The fungus arrives at this location after initiating and synchronizing its intercellular growth with the growth of tall fescue flower shoots early in the spring. As stated above, the systemic hyphae of the endophyte of tall fescue are similar to other endophytes (Fig. 1a).

The fescue seed is the only demonstrated means by which this parasite is dispersed and from which infection is accomplished. In the dormant seed the fungus is located in the aleurone layer, mainly in the vicinity of the embryo, but it has not been observed in the embryo. However, embryo infection apparently takes place shortly after germination, as Lyons and Bacon (26) have observed the fungus in the first internode of the emerging shoot within 2 days of germination. The coleoptile does not become infected at all and the first leaf and subseqeunt leaves are not infected until after sheath differentiation occurs.

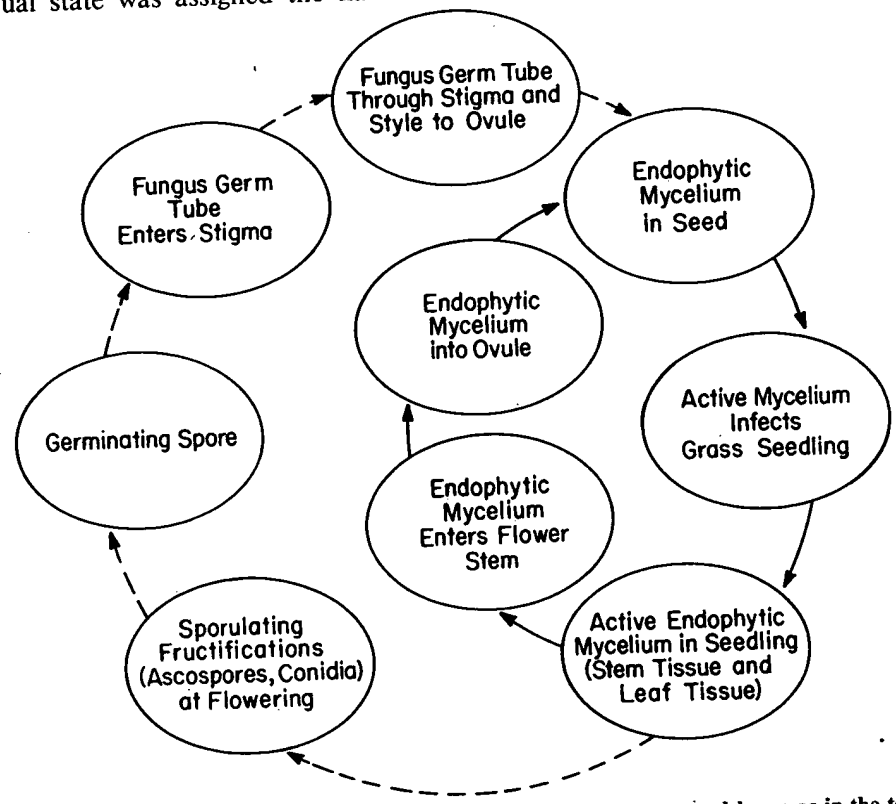

Fig. 2. Generalized life cycles of pasture grass endophytes. *Solid arrows*: endophytes that are seed-borne as in the tall fescue endophyte and in cleistogamous Balansiae-infected seeds; *Broken arrows*; transmission of endophytes to uninfected plants via spores from infected plants.

Among a seedling population, infection of the leaves occurs within a 2-week period, usually commencing 8 to 10 days after germination, although this can be affected by environmental factors and possibly seed age and storage conditions. The fungus loses its viability in the seed over a period of about 1 yr unless the seed is stored at cold temperatures. Presently, the degree to which a seedlot is viably infected is determined reliably only by using a grow-out procedure in which the percent of infected seedlings produced by the seedlot is assayed. When using typical staining and microscopic methods for detecting the fungus in the seedlings, it is impossible to actually detect the hyphae until the time when leaf infection occurs and the fungus proliferates. Thus, the grow-out procedure requires a minimum of 3 weeks or longer.

The dissemination of the parasitism via seed serves an important and similar dispersal mechanism (49) as that of the fungal spore, and there may be advantages. Fungi of the Clavicipitaceae family are noted producers of both asexual and sexual spores that are viable for only a few weeks. Thus, external infection by species of these two genera must take place within a short period. In seeds, the endophyte of tall fescue may be viable for an average period of 1 yr, so the potential for dissemination and infection have been increased. The tall fescue endophyte is located within the leaf sheath and not the blade, and a similar distribution has been reported for the endophytic fungus in perennial ryegrass (14). In contrast, systemic hyphae of other members of the Balansiae (species of *Balansia*) are distributed throughout the leaf blade and sheath, but unlike the fescue endophyte, hyphae of these species slightly alter host cell morphology (38, 45).

HOST AND GEOGRAPHIC DISTRIBUTION

Endophyte in Weeds

There are 17 species of *Balansia*, of which 13 are endemic to the USA and other countries of the western hemisphere (11). Eleven of the 13 American species occur on nine tribes of the Gramineae, representing 125 perennial grass species (Table 1). The remaining two *Balansia* species parasitize the Cyperaceae. The panicgrass tribe (*Panicum* sp.) (Paniceae) contains the largest number of recorded hosts for the *Balansia* species. Grasses belonging to this tribe include both temperate and tropical species with a very wide geographical distribution. Several grass species of this large tribe are extremely obnoxious weeds, and therefore should be examined for infection by *Balansia* spp. The cited records for the *Balansiae* have established that infected grasses occur in this continent within a range of states from Florida in the South to New York andMassachusetts in the north, and west from Texas and Missouri to North Dakota. Since there has been no systematic large-scale survey of grasses for these elusive fungi, the number of host plants infected may be more extensive than reported.

Table 1. The distribution of the world species of *Balansia* among the grass tribes.

Tribe†	Distribution *Balansia* species
	%
Agrostideae	23
Andropogonease	23
Bambuseae	15
Chlorideae	15
Festuceae	15
Hordeae	8
Oryzeae	8
Paniceae	54
Zizanieae	8

† There are five tribes of grasses that are not reported as host: Aveneae, Melinideae, Phalarideae, Tripsaceae, and Zoysieae.

Almost all of the grass hosts of the *Balansia* species are considered weeds of varying forage quality. Some of the weed grass hosts have been domesticated and developed into cultivars with improved palatability and nutritional quality (19). One host, bermudagrass [*Cynodon dactylon* (L.) Pers.], is an economically important forage grass in the South. Other host grasses that have been used for forages are *Danthonia, Lolium, Poa*, and *Calamagrostis*. The cultivated as well as noncultivated grasses have value throughout the USA in low-rainfall areas and rangelands. Due to the vigor and recovery from excessive grazing, the distribution of weed grasses in pastures is extensive and cattle graze infected plants, consuming enough to show toxicity signs.

The density of any one weed grass could reach 40% within a poorly managed pasture, or a pasture grass growing in its marginal zone, especially if the pasture grass is a cool-season species. For example, in a tall fescue pasture in Georgia, smutgrass (*Sporobolus poiretii* Roem. and Schult.) is a major weed occurring during the summer months, and for lack of desirable forage is grazed by cattle. Smutgrass is parasitized by *B. epichloe* and in some pastures as much as 60% of this grass is infected. Other weed grasses growing during the summer months in fescue pastures in Georgia are species of lovegrass (*Eragrostis*), panicgrass, and broomsedge (*Andropogon*). The density of the weed grasses depends on the terrain, climatic conditions, management, grazing pressure, and the vigor of the forage grass. The majority of the hosts of the *Balansia* are warm-season perennial grasses and sedges, appearing most prevalent throughout summer and into early fall. Cattle grazing pastures during summer are therefore exposed to a variety of weed grasses, which, if infected, could expose the animal to a variety of toxins.

Each weed grass species, if infected, is parasitized by one *Balansia* species; two species of *Balansia* do not occur on the same tiller. However, a grass species can serve as host for several of the Balansiae. For example, *Andropogon scoparius* Michx. is a host of *B. epichloe* and *B. henningsiana* (Moll.) Diehl, and *Eragrostis hirsuta* (Michx.) Nees is a host for *B. epichloe* and *M. atramentosa*. Double infection of a single grass species by different genera of the Balansiae does occur. Thus, single plants of *Panicum anceps* Michx. are oftentimes infected with *M. atramentosa* and *B. henningsiana* (24, 38). Double infection of the same host by these two parasites reflects the difference in ecological niches of their infection patterns. In *B. henningsiana*, the mycelium is intercellular, and the fruiting stromata form on the abaxial leaf sheath; in *M. atramentosa* the mycelium is superficial and localized

into adaxial stromata on the leaf blade (24). The occurrence of two or more hosts of a fungus species does not mean that all of the recorded hosts will be infected. In certain pasture situations, broomsedge may be parasitized by *B. henningsiana* but another host for this fungus growing in close proximity to infected broomsedge will be uninfected. In another location, the host infection pattern may be reversed. Similar observations have been reported by Diehl (11). This suggests that disseminaiton is by seed and that there may be host specific biotypes of the fungi.

Endophyte in Tall Fescue

The seed-borne nature of the parasite accounts for the widespread occurrence of infected tall fescue in the USA. Tall fescue is considered to have originated in northern Europe, and indeed infected fescue has been reported from this location (44). Tall fescue is grown on 15 million ha and, based on a conservative estimate, three-fourths of these acres is infected. The use of infected European ecotypes to improve tall fescue, the permanent nature of fescue pastures, and the planting of freshly harvested seed greatly assisted in the widespread infection pattern of fescue pastures. In addition to tall fescue, 15 other species of fescue are reported as being infected (22, 50); several of these are endemic to the USA, and two of these are annual fescues. The infection of the remaining 18 U. S. species of fescue by an endophyte has not been determined. It appears that the genus *Festuca* may be uniformly parasitized by the endophyte regardless of the grass species' geographical origin and its annual or perennial growth habit. Collectively, the range of infected fescue species include all states of the continental USA (20).

Ecological Significance

The pathogenic effects of the Balansiae are usually minor and sometimes absent. In contrast, it appears with some species that infection confers favorable attributes on the host, which may be important in its growth and survival. These various attributes may include: altered growth habit and increased vigor, insect resistance, and mammalian toxicity (9, 15, 37).

Altered growth habit, increased vigor, or both apparenty occurs in a number of endophyte-infected grasses. For example, *Agrostis tenuis* Sibth. was described as having a more prostrate habit and spreading more vigorously when infected with *Epichloe typhina*. The tall fescue endophyte, which is closely related to *E. typhina*, but does not sporulate on its hosts, also appears to increase the vigor of its hosts (37). Diehl (11) similarly observed that *Cenchrus echinatus* L., when infected with *B. obtecta*, was larger than its uninfected counterpart, and furthermore that with many grasses in older pastures infection with *Balansia* spp. became more predominant. This latter observation suggests that infected grasses have a competitive advantage. Thus, it is interesting in this regard that Clay (9) found *D. spicata* infected with *Atkinsonella hypoxylon* (Pk.) Diehl superior to uninfected plants when grown in competition with *Anthoxanthus odoratum* L.

Where sterility does occur as a result of infection, it seems that this represents a change in the host whereby sexual variation and dissemination by seed are sacrificed for an advantage in growth and survival over neighboring plants. In cases where plants are partially or entirely sterilized, some of the growth advantage may result from the fact that host assimilates normally diverted to the flowering and reproductive processes are now available for continued vegetative growth. It is probable that hormonal effects are involved, since growth habit can be altered and increased vigor occurs in infected grasses that are not sterilized (*see* Host Responses and Toxins below).

It has recently been shown that endophyte infection can increase insect resistance in ryegrass (15). Tall fescue is noted for its resistance to insects and other pests. Studies on the significance of insect resistance in infected tall fescue pastures have not been conducted. Nevertheless, in ryegrass uninfected stands frequently suffer serious damage to the Argentine stem weevil in new Zealand (15, 23). To date, there is no evidence that infection by other species of the Balansiae confers resistance to any grasses, but studies to this effect are warranted in light of the potential ecological significance of this phenomenon. Since evidence concerning such resistance in tall fescue suggests that the host plays an important role, it may be that only in some grasses will this condition be expressed, even when different grasses are infected by the same fungus species.

HOST RESPONSES AND TOXINS

Weed Grasses and Toxins

Information on the effects of the *Balansia* on the host's morphology are largely observational and these effects probably are not caused by a single mechanism in all of these diseases. Although no necrotic lesions are produced, chlorotic lesions are eventually produced in the area beneath and immediately surrounding the stromata possibly due to shading. After a stroma matures on a leaf, it disintegrates, the chlorotic area becomes less chlorotic. Other symptoms of infection include lack of flower and seed development, dwarfness, and deformation of the flag leaf.

The dwarfed and fasciated condition of infected grasses possibly result from one or a combination of the following: (i) nutritional stresses on the plant; (ii) a physical barrier such as the development of a stroma at the time of flowering; and (iii) the production of either plant growth inhibitors or altered metabolism of plant growth regulators. Plant inhibitors and altered hormone metabolism are likely possibilities when we consider that hyperauxiny in some parasitized plants is well established and that each of the major groups of plant hormones has been isolated from pathogenic fungi. We have demonstrated that *B. epichloe* produced indole acetic acid, indole ethanol, indole acetamide, and several other unidentified indole derivatives in culture (30, 34). These studies established with wheat coleoptile (the biological assay) that an increase of up to 63% in growth occurred at low concentrations and complete inhibition of growth at high concentrations. The in situ production and effects of these hormones on the plant and the overall effects of C and N assimilation in infected plants have not been determined, but are the subjects of current research.

Table 2. Alkaloid production in culture by species of *Balansia*.

Fungus	Ergot alkaloids	References	Indole alkaloids†	References
B. epichloe	Chanoclavine I	4, 5, 32	Erytho 1-(3-indolyl)pro-pane-1,2,3-triol	34, 35
	Isochanoclavine I	32		
	Agroclavine	4, 5, 32		
	Elymoclavine	32	Threo 1-(3-indolyl)pro-pane-1,2,3-triol	34, 35
	Penniclavine			
	Ergonovine	4, 5, 32	Indole acetic acid	30
	Ergonovinine	4, 5, 32	Indole ethanol	30
	6,7-Secoagroclavine	30	Indole acetamide	30
B. claviceps	Chanoclavine I	5, 32		
	Ergonovine	5, 32		
	Ergonovinine	5, 32		
B. henningsiana	Chanoclavines	4, 5, 31		
	Dihydroelymoclavine	31		
B. strangulans	Chanoclavines	5, 31		
	6,7-Secoagroclavine	31		

† Only *B. epichloe* were examined for indole alkaloids (nonergot type).

Table 3. Host range and geographic distribution of *Balansia epichloe* (38).

Host	Location	Reference
Agrostis alba	GA	18
Andropogon scoparius	NC	11
Andropogon sp.	AL, SC, MO	11
Calamogiostis canadensis	WI	11
Calomogrostis inexpansa	ND	11
Calamogrostis sp.	WI	11
Chasmanthium laxum	TX	38
Chloris petraea	Cuba, PR, FL	11
Chloris sp.	FL	47
Chloris sp.	Mexico	11
Ctenium aromaticum	NC	11
Ergrostis capillaris	AL, SC	11
	FL, GA, VA	47
Eragrostis hirsuta	GA	11
	AL, FL, VA, SC	47
Eragrostis refracta	AL, FL, GA, VA	11
	SC	47
Ergrostis secundiflora	GA	4
Eragrostis trichocolea	FL	11
Eragrostis sp.	SC, FL	11
Gymnopogon ambiguus	GA, NC, VA	11
Oryzopsis asperifolia	PA	47
Panicum agrostoidea	MO	11
Panicum anceps	GA	7
Panicum sp.	MS	11
Sporobolus indicus	AL, MS, Brazil	11
Sporobolus poiretii	AR, LA, MS, AL, GA, NC FL, Costa Rica, Brazil	11
	VA	47
Sporobolus sp.	AL, MS, FL, Brazil	11
Thrasya petrosa	Brazil	11
Triodia flava	AL, KS	11

Generally the relationships of the Balansiae to a grass are fungus specific. Oftentimes infected plants are sterile and any seeds that may be produced do not germinate. There are two ways that sterility is imposed on plants. One is by the mechanical binding of fungus tissue to developing inflorescences as represented by *B. claviceps* and other members of the subgenus *Eubalansia* Diehl (Fig. 1b). The second is through hormonal and other biochemical controls. This type is represented by the remaining species of *Balansia* belonging to the subgenus *Dothichloe*, and, where host flower development is repressed. However, the effects of a fungus in the subgenus *Dothichloe* Diehl on its grass host are not absolute, which suggest a complex biochemical interaction. For example, one member of this subgenus, *B. epichloe*, produces total sterility of smutgrass, but two species of lovegrass infected with this same fungus produce flowers and set viable seed.

Paralleling this flowering phenomenon is the production of ergot alkaloids by this fungus. All isolates from smutgrass produce ergot alkaloids as opposed to the lack of production of these toxins from isolates from the lovegrasses (4, 33). These two genera of grasses belong to the same tribe, but unlike the lovegrasses, smutgrass is not a native grass of the western hemisphere and was introduced from tropical Asia (20). The Balansiae have not been reported in tropical Asia; therefore, smutgrass probably became infected after its introduction in the USA. The resulting toxins and lack of flowering suggest that this parasitism is not as compatible as that in the native hosts. According to Diehl (11), there are 18 host species of foreign origin and of these, five are important forages: *Axonopus compressus* (Swartz) Beauv., *Bambusa arundinacea* Retz., *Cynodon dactylon* (L.) Pers., *Setaria palmifolia* (Koen.) Stapf., and *Sorghum vulgare* Pers. (*S. bicolor*). The distribution of toxic isolates of Balansiae from introduced hosts is unknown, but *C. dactylon* (bermudagrass) periodically becomes toxic, and produces tremors in cattle, the signs of which are identical to those of convulsive ergotism (12, 36). The etiological agents are considered ergot alkaloids, but only on one occasion has a species of *Claviceps* been associated with toxic bermudagrass (36). Unfortunately, toxic bermudagrass was not examined for members of the Balansiae, but *B. obtecta* Diehl (Fig. 1g) is reported as a parasite of this grass (11).

The chemically identified toxins produced by *Balansia* species are ergot alkaloids identical to those produced by species of *Claviceps* (Table 2). The possibility of additional types of toxins should be explored. The bioproduction and chemical identification of the alkaloids produced by members of the Balansiae involved a series of fermentations by isolates on laboratory media (4, 5). We used *B. epichloe* as a model for developing laboratory procedures necessary to determine ergot alkaloid production, and have included its host range and geographic distribution (Table 3) (38). This fungus has the highest frequency of occurrence among the various grass tribes and several isolates are producers of high levels of ergot alkaloids. The fermentation procedure allowed us to conclude that a species of *Balansia* produced similar toxins in vitro and in vivo, and that nonproducing isolates in vitro are nonproducers in vivo (5).

The specific requirements for a host, fungus, or both,

which result in the production of ergot alkaloids in the plant are unknown, but possibly the degree of compatibility, as suggested above for smutgrass, might be a key factor. Strains of the fungus vary in their in vitro production of alkaloids, and host produced precursors might also be involved in the final in vivo response. It has been determined that tryptophan is a requirement for the laboratory culture of ergot alkaloids. This compound is not found in high levels in the free amino acid pool of several grasses; therefore, it could be one of the rate-limiting factors. In addition to tryptophan, there are probably other limitations. The relationship of the host with a genetic strain of the fungus in the production of ergot alkaloids is illustrated by data showing that isolates of the same species of *Balansia* only produced alkaloids on certain hosts (Table 4). In one pasture, 59% of the isolates of *B. henningsiana* from broomsedge produced alkaloids as opposed to the production of alkaloids by 100% of the isolates of *B. epichloe* from smutgrass. Based on the distribution and density of broomsedge, this indicates that *B. henningsianna*-infected broomsedge in a pasture would not contribute as much ergot alkaloids to the cattle diet as *B. epichloe*-infected smutgrass. In other locations, broomsedge might not contain ergot alkaloids at all. We do not want to suggest that certain *Balansia*-infected grasses are not toxic, only that they do not contain ergot alkaloids. Other classes of toxins might be produced, but for a lack of a valid bioassay, these toxins have not been detected. Indeed, *Balansia*-infected grasses that do contain ergot alkaloids might also contain other types of toxins. Moreover, the involvement of a fungus-plant species in cattle toxicities is confounded because two to three species of *Balansia*-infected weeds are oftentimes found in one location.

Tall Fescue and Toxins

The review by Yates (53) considered the various classes of compounds isolated from fescue relative to grass toxicity prior to 1984, while an earlier review (54) is of considerable historic significance since it defined the nature of the toxicity problem. Of current interest is the finding of ergot alkaloids in infected tall fescue that are identical to those produced by the fescue endophyte in culture. The purpose of this review is not to ignore the early isolated compounds nor promulgate their unrelatedness to the fescue toxicity syndrome, but rather to briefly review a potent class of ergot alkaloids never reported in fescue, whose very structure suggests that they are major candidates for toxicity. These recently isolated ergot alkaloids are totally unrelated to those alkaloids reported earlier (53, 54) as being involved in the toxicity syndrome, e. g., perloline and loline.

The ability of the endophyte of tall fescue to produce ergot alkaloids in culture was demonstrated in 1979 (32), but it was not until recently that these same alkaloids were isolated from leaf blades and sheaths of infected fescue (16, 27). Alkaloids are not found in noninfected tall fescue. It is not known if the other species of fescue parasitized by endophytes also contain ergot alkaloids. The endophyte of tall fescue produces in addition to the clavine group characteristic of *Balansia*-weed infections, the much more biologically active group of alkaloids, the ergotamine peptide alkaloids (Table 5). These fescue ergot alkaloids are distributed within the leaf blade and sheath (27) and seed (16). The distribution of ergot alkaloids in fescue indicate that there are quantitative differences. Since there is no fungus tissue in the blade, it is assumed that the ergot alkaloids are produced by the fungus and translocated to the blade. This assumption is strengthened when we consider that the isolated fungus produces ergot alkaloids in culture. The factors responsible for biosynthesis of the ergot alkaloids and their translocation may be related to the season, the nutrient status of the plant such as soil nitrogen, or both. It has been observed that high levels of N appear to increase fescue toxicity (16, 54). Stressing and nonstressing environmental conditions may also influence the level of ergot alkaloid synthesized.

TOXICITY IN CATTLE

The toxicity signs of ergotism in cattle include reduced weight grain and milk production, emaciation, elevated temperatures, reproductive problems (failure to cycle or conceive and abortions), gangrene of the extremities, and nervous or palsy in the flank region. The degree to which any of these signs dominate in a herd would depend on the amount and chemical spe-

Table 5. Total ergot alkaloid and pepetide ergot alkaloids in leaf sheaths and leaf blades of 15 endophyte-infected tall fescue samples collected from field and greenhouse.

Alkaloid fraction	Range of concentrations	
	Leaf blade	Leaf sheath
	mg kg^{-1}	
Total ergot alkaloids†	0.4–3.5	0.7–22.0
Peptide ergot alkaloids‡	0.1–0.5	0.3– 4.0
Ergovaline‡	0.1–0.4	0.3– 3.5
Ergosine‡	<0.05	<0.2
Ergonine‡	<0.05	≤0.2

† Concentrations determined colorimetrically with a modified Van Urk's reagent (5) and ergonovine as standard.
‡ Concentrations determined by tandem MS with ergovaline as standard.

Table 4. The percentage of isolates of *B. epichloe* and *B. henningsiana* producing four major alkaloids from different grass species.

Fungus species†	Grass species	Percentage isolates producing major alkaloids				Percentage producing alkaloids
		Chanoclavine (I)	Ergonovine	Agroclavine	Ergonovinine	
B. epichloe	*S. poiretii*	100	63	42	63	100
B. epichloe	*Eragrostis hirsuta*	nd	nd	nd	nd	nd
B. epichloe	*E. secundiflora*	nd	nd	nd	nd	nd
B. henningsiana	*Andropogon virginicus*	59	35	nd	35	59
B. henningsiana	*Panicum tenerum*	nd	nd	nd	nd	nd

† A total of 43 isolates of *B. epichloe* from *S. poiretii* and 34 isolates of *B. henningsiana* from *A. virginicus* were collected from one location in Georgia. Alkaloids were not detected (nd) in 15 isolates from *E. hirsuta* collected from Georgia, 5 isolates from *E. secundiflora* collected in Texas, 14 isolates from *P. tenerum* collected from Georgia, and 2 isolates from *P. tenerum* collected from Missouri.

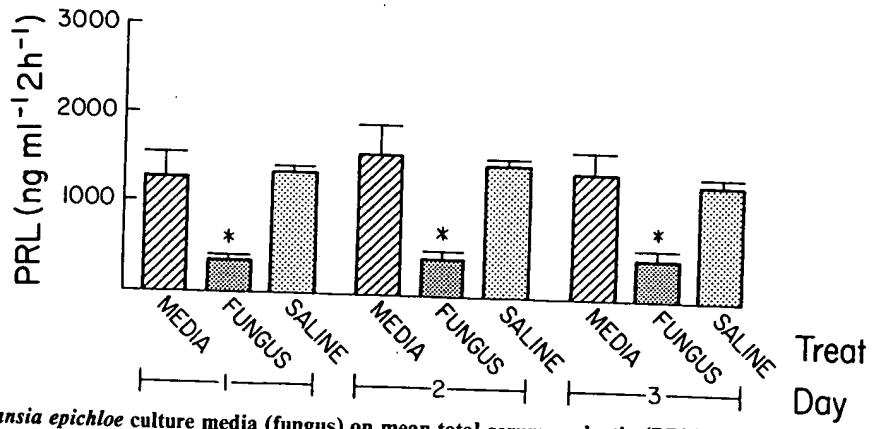

Fig. 3. The effect of *Balansia epichloe* culture media (fungus) on mean total serum prolactin (PRL) concentrations in lactating Holstein cows (2 cow group^{-1}). Mean PRL is expressed as area under the curve after challenging with thyrotropin-releasing hormone (200 μg cow^{-1}). Blood was collected at 15-min intervals for 2 h and assayed for PRL using a radioimmunoassay technique. Brackets indicate standard deviation; media (uninoculated) and saline are controls. *Significantly different ($P<0.05$) from media-treated and saline-treated groups. Data from Wallner et al. (48) with permission of the authors.

cies of alkaloids consumed. Ergonovine and agroclavine are the most toxic of the alkaloids produced by the species *Balansia*. Many cattle toxicity signs are similar to those experimentally produced in laboratory animals (8), sheep (*Ovis aries*) and cattle (17, 46, 52), which suggests that the alkaloids produced by endophytic fungi could in various combinations and concentrations produce signs shown by cattle grazed on *Balansia*-infected weeds and endophyte-infected tall fescue. The effects of cultures of *B. epichloe* culture isolated from infected smutgrass on serum prolactin of cows have been determined (48). This study established that the fungus depressed serum prolactin levels (Fig. 3). In addition, there was a decrease in the milk-induced rise in serum prolactin concentration, but milk production was not affected in lactating cows. However, additional studies indicated that a decrease in serum prolactin 1 to 2 days before the onset of lactation would cause a decrease in milk production during subsequent lactation (1, 2). This suggests that cows consuming *B. epichloe*–infected weed grasses during gestation would have decreased milk production after parturition. The chronic effects of various levels of ergot alkaloids on cattle are unknown, but these levels could cause the reduced weight gain, directly or indirectly, as reported by Smith et al. (46).

The seasonal production of ergot alkaloids throughout the growing period and distribution within infected grasses has yet to be determined. It is unknown when the ergot alkaloids appear in the grass-fungus association, although we have found significant concentrations in infected tall fescue sampled for 5 different months within three seasons of the year (C. W. Bacon, 1984, unpublished). An analysis of field grown infected smutgrass in late summer when black stromata were evident demonstrated that leaves of this weed grass contained 17 mg kg^{-1} total alkaloids (dry weight) (5). The distribution of the toxins in the smutgrass leaf blade, sheath, and root was not determined, but data on the distribution of toxins in fescue indicate only quantitative differences. The major alkaloids identified from smutgrass were chanoclavine (I) (16.0 mg kg^{-1} dry wt), and ergonovine (0.48 mg kg^{-1} dry wt). There were several other alkaloids in the leaves, but they were not identified because of their small concentration. In well-managed pastures, weeds will comprise only a small portion of the grass community and even if they are infected by an alkaloid-producing strain, preferential grazing may not include them in the cattle diet. Nevertheless, if it is on a daily basis, cattle grazing on *Balansia*-infected smutgrass would result in a considerable intake of alkaloid. For example, a 500-kg cow consuming only smutgrass would consume approximately 10 kg dry wt day^{-1} of forage; this cow would be ingesting a minimum of 170 mg crude ergot alkaloids day^{-1} or 4.8 mg ergonovine day^{-1}. While cattle grazing on tall fescue would consume less total alkaloids, it must be remembered that the class of alkaloids produced by this endophyte is considerably more toxic, and their physiological effect is very broad. Moreover, as tall fescue is a major pasture species, the likelihood of cattle consuming only this grass, thereby receiving chronic levels, is a reality.

Unlike the other group, the ergotamine peptide alkaloids produced by the fescue endophyte are characterized as having a wide spectrum of activities that include excitatory reflexes, hyperthermia, salivation, vomiting, serotonin antagonism, reduction of serum prolactin levels and lactation, abortions, uterine contraction, and vasoconstriction (8). These are signs of the fescue toxicity syndrome, and several of these signs are also produced by the clavine group of ergot alkaloids (8, 28).

The major peptide ergot alkaloid produced by the endophyte of fescue is ergovaline (Table 5), but the effects of it and the remaining peptide alkaloids, ergosine and ergonine, on cattle have not been demonstrated. However, it has been established that ergotamine, the parent alkaloid of the class to which these two belong, produced signs in cattle and sheep similar to those seen in cattle grazing tall fescue (17, 52). The account by Woods et al. (52), relates the effects of air and cattle body temperatures to variations in gangrenous ergotism produced by ergotamine in cattle. This report also determined that the ruminant stomach is extremely efficient in extracting alkaloids from plant materials.

A comparative study of ergovaline and ergosine with

ergotamine on nidation in rats (*Rattus* sp.) indicated that not only are they similar to ergotamine, but they are 2 to 4 times more active (13), and have lower LD_{50} values (8). Thus, the physiological effects of ergovaline and ergosine are expected to be just as broad but more toxic than ergotamine. The laboratory studies suggest that the acute and chronic effects of these biologically active peptide alkaloids alone, and in combination with clavine alkaloids, should be determined in cattle.

CONCLUSION AND AGRONOMIC IMPLICATIONS

Research on weed grass endophytes of pasture grasses has established that ergot alkaloids are produced by fungi other than *Claviceps*. The alkaloids are identical to those produced by species of *Claviceps*, which are ovarian parasites of grains. This suggests that the potential for alkaloid biosynthesis was conserved during evolution by various members of the family Clavicipitaceae. The Balansiae, unlike species of *Claviceps*, produce ergot alkaloids which are distributed throughout the plant, especially in the leaves. Thus, the management practice of mowing grass seed heads as recommended to prevent *Claviceps* infection would not be beneficial in removing endophyte-produced toxins and fungi from grasses. Mowing weed grasses might prevent the spread of the parasite by not allowing infection to take place, a priori, via seeds from noninfected plants. This is an absolute requirement for tall fescue pastures where the level of infection increases due to the established seed-borne nature of the parasite. For example, it is presently recommended that fungus-free seeds be used to obtain endophyte-free tall fescue pastures. These seeds are usually 99 to 95% fungus free, but the level of infection will in succeeding years increase to a much higher level if seeds are allowed to develop. Indeed, preliminary field observations indicate that depending on the prevailing environmental conditions, infected plants produced more seed than healthy plants.

Several of the endophytic fungi are parasites of weed grasses that invade and successfully compete with major pasture grasses. A fescue pasture can contain infected fescue grasses and infected weed grasses. In cool-season grasses the density of weed grasses and the extent of their infection will be evident during the stressful period, i.e., summer. Weed grasses belonging to the panic grass tribe (Paniceae) should be examined for infection as most species of it serve as host for *Balansia* spp. Pasture grass management designed to prevent the growth of weed grasses should be emphasized and in terrains where a good stand of desirable forage cannot be achieved, the vigorously growing nonhost species should be planted. The selected weed grass species should also have the ability to withstand grazing conditions. *Balansia*-infected weed grasses that are established in pastures should be destroyed and a desirable forage grass replanted. There is no known systemic fungicide that will permanently control this group of fungi from established pastures. Recently some control of the fescue endophyte has been reported with propiconazol {1-[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-ylmethyl]-1*H*1,2,4-triazole} and triadimefon [1-(4-chlorophenoxy)-3,3-dimethyl-1-(1*H*-1,2,4-triazol-1-yl)-2-butanone] applied to established pastures (51). The cost of this control, which lasts for 1 yr, may prevent this method from being practical. Moreover, the use of a fungicide may have no value, since depending on the endophyte there is always the risk of reinfection. The key here is to remove the infected host and replace it with a nonhost species or noninfected forage grass. Alternatively, a grass host for the *Balansia* can be used since ergot alkaloid accumulation by an infected grass species is host and fungus related. Infected but nontoxic fescue species may also be used if future research establishes that they exist.

The potential ecological advantages to be derived by certain grasses from infection by some of the Balansiae are apparent from the above discussion. However, depending on the grass or the effect, infection may or may not be desirable to the farmer. Thus, while infection of tall fescue with the endophyte may possibly confer a growth advantage and better insect resistance on the grass, it is unlikely that the advantages would be worth the losses arising from the resulting toxicity. This is especially true since insects are only rarely of consequence on tall fescue in the USA. Furthermore, the growth advantage may be negated in a well-managed pasture. The dilemma that might be faced is very clear, as in the case of perennial ryegrass in New Zealand where infected pastures suffer little damage from the Argentine stem weevil (*Listronotus bonariensis* Kuschel), but may be highly toxic, causing the condition in cattle known as ryegrass staggers. Conversely, uninfected pastures may be so severely affected by the stem weevil that they do not provide adequate grazing. Most of the Balansiae do not present this kind of dilemma as they are present mainly on weedy grasses and as such are of no potential economic benefit. It is their potential as causal agents of toxicity that is of utmost concern.

In conclusion, the association of endophytic fungi with grasses is widespread, occurring on all but four of the grass tribes. Without regard to any beneficial effects derived by each member from the association, infected grasses are potential hazards to cattle. Ergot pasture grass toxicities should not necessarily be attributed to infection of the major forage grass established by the farmer nor to infection by the genus *Claviceps*; the involvement of endophyte-infected grasses growing in association with pasture grasses should be explored.

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TI Ergot alkaloid biosynthesis by isolates of Balansia epichloe and B. henningsiana
AU Bacon, Charles W.; Porter, James K.; Robbins, Joe D.
CS Richard B. Russell Res. Cent., USDA, Athens, GA, 30613, USA
SO Can. J. Bot. (1981), 59(12), 2534-8

TI Conformations of the ergot alkaloids chanoclavine-1, aurantioclavine, and N-acetylaurantioclavine [Claviceps purpurea, Fungi].
AU Sakharovskii, V.G.; Aripovskii, A.V.; Baru, M.B.; Kozlovskii, A.G.
AV DNAL (QD241.K453)
SO Chemistry of natural compounds., Sept/Oct 1983 (pub. 1984) Vol. 19, No. 5. p. 626-627

CAS

TI Peptide-type ergot alkaloids produced by Hypomyces aurantius
AU Yamatodani, Saburo; Yamamoto, Isao
CS Kobe Women's Junior Coll., Kobe, 650, Japan
SO Nippon Nogei Kagaku Kaishi (1983), 57(5), 453-6

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Biosynthesis of ergot alkaloids. Mechanism of the conversion of chanoclavine-I into tetracyclic ergolines
AU Floss, Heinz G.; Tcheng-Lin, Marie; Chang, Ching-Jer; Naidoo, Bala; Blair, Garre E.; Abou-Chaar, Charles I.; Cassady, John M.
CS Dep. Med. Chem., Purdue Univ., West Lafayette, Indiana, USA
SO J. Amer. Chem. Soc. (1974), 96(6), 1898-909

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SO J. Agric. Food Chem. (1981), 29(3), 653-7

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TI ALKALOIDS FROM THE FUNGUS CLAVICEPS-SP IBPM-F-401.
AU KOZLOVSKII A G; ARINBASAROV M U; SOLOV'EVA T F; ADANIN V M; GRIGOROV I; ANGELOV T I; SLOKOSKA L S; ANGELOVA M B
CS INST. BIOCHEM. PHYSIOL. MICROORG., ACAD. SCI. USSR, PUSHCHINO, USSR.
SO PRIKL BIOKHIM MIKROBIOL, (1980) 16 (4), 569-577.

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AU BACON C W; PORTER J K; ROBBINS J D
CS US FIELD CROPS LAB., US SCI. EDUC. ADM., R. B. RUSSELL AGRIC. RES. CENT., ATHENS, GA. 30604, USA.

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DOES DECREASED MOWING FREQUENCY ENHANCE ALKALOID PRODUCTION IN ENDOPHYTIC TALL FESCUE AND PERENNIAL RYEGRASS?

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Abstract—Tall fescue, *Festuca arundinacea*, and perennial ryegrass, *Lolium perenne*, are widely infected with fungal endophytes (*Neotyphodium* spp.). The symbiosis between plant and fungus leads to synthesis of alkaloids that have been shown to be either toxic or act as feeding deterrents against insect pests. As cultural practices have the potential to regulate production of plant secondary metabolites, we evaluated the influence of mowing frequency on the levels of major alkaloids in tall fescue and perennial ryegrass in the greenhouse. Tall fescue and perennial ryegrass maintained in 15-cm-diam. pots were cut to 5-cm height weekly or biweekly. Samples were taken monthly and the alkaloids extracted and analyzed by reverse-phase LC-MS. In tall fescue, ergovaline, ergonovine, and ergocristine were identified, whereas only ergocristine was identified in perennial ryegrass samples. In tall fescue, we observed a trend showing higher levels in samples cut biweekly than in those cut weekly. A similar pattern was seen in some putative alkaloids that were not identified. In perennial ryegrass, ergocristine and two putative alkaloids followed a pattern similar to that of alkaloids in tall fescue. A survey of a few samples of perennial ryegrass using extractions specific to peramine and lolitrem B yielded evidence suggesting their presence as well as several other identified alkaloids. These data support the hypothesis that decreased mowing frequency enhances alkaloid production/accumulation in tall fescue and perennial ryegrass.

Key Words—Fungal endophytes, *Neotyphodium coenophialum*, *Neotyphodium lolii*, alkaloids, tall fescue, *Festuca arundinacea*, perennial ryegrass, *Lolium perenne*, mowing frequency.

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INTRODUCTION

Tall fescue, *Festuca arundinacea* Schreb, and perennial ryegrass, *Lolium perenne* L., are widely infected by the fungal endophytes *Neotyphodium coenophialum* Morgan-Jones & Gams and *Neotyphodium lolii* Latch, Christensen & Samuels, respectively. These mutualistic symbioses lead to production of several alkaloids that provide infected plants with chemical defenses against herbivory (Prestidge et al., 1982). Livestock feeding on the endophyte-infected grass suffer toxicoses from alkaloid ingestion (Porter et al., 1974; Hoveland, 1993). At least 15 species of insects are affected by endophyte alkaloids (Siegel et al., 1990; Rowan et al., 1990; Breen, 1993). This chemical defense also extends to other organisms including nematodes (West et al., 1988) and fungi (White and Cole, 1985).

Tall fescue and perennial ryegrass are widely used turf grasses in home lawns, golf course fairways, driving ranges, and public parks. They sometimes require application of pesticides to manage insect pests. Heavy use of chemical pesticides in urban environments leads to increased health risks to pets, children, and adults. Not surprisingly, the use of many organophosphate and carbamate pesticides has been banned by the United States Environmental Protection Agency or is under review in order to implement the Federal Food Quality Protection Act. However, this has, led to a more intensive use of other pesticides, such as neonicotinoids, that are deemed safe, relative to organophosphates.

The potential of endophyte-mediated resistance offers a promising avenue of investigation (Clay, 1989). If pesticide use in urban environments could be reduced through cultural practices (fertilization, irrigation, clipping), this could lead to health and economic benefits for homeowners and managers of golf courses and recreational parks. There are indications that the alkaloid levels in endophyte-infected turf grasses can be manipulated by management practices. For instance, clipping has been shown to reduce alkaloid content in tall fescue (Belesky and Hill, 1997). This occurred as a function of nonstructural carbohydrates that were channeled from alkaloid synthesis to production of leaf matter. This led us to hypothesize that decreased mowing frequency would result in an increase in the level of endophytic alkaloids. Here we present results of a greenhouse study on the effect of mowing frequency on levels of major alkaloids produced in tall fescue and perennial ryegrass.

METHODS AND MATERIALS

Grass Management and Mowing Frequency. Six 15-cm-diam. cores of established stands of endophytic tall fescue, *F. arundinacea*, and endophytic perennial ryegrass, *L. perenne*, were taken from field plots established in 1999 at the Ohio Agricultural Research and Development Center. The cores were placed into 15-cm-diam. pots and transferred to a greenhouse in the fall of 2000. The

percentage of tillers infected by the endophyte was in the range of 30–100% with an average of 58% for the tall fescue, and 50–90% with an average of 77% for the perennial ryegrass (Grewal, unpublished data). Plants were maintained at 21°C day and 16°C night and a 16L:8D light cycle. Daylight was supplemented with artificial lighting as required. Plants were watered as a needed, and weekly applications of Peters Professional fertilizer [(20-20-20 N-P-K) (Scotts Company, Maysville, Ohio, USA] was provided. The plants were maintained in greenhouse for four weeks before sampling was initiated. The grasses were cut to 5-cm height either weekly or biweekly (every other week).

Sampling. Triplicate samples were collected monthly, starting one month after the first treatment. Sampling was limited as much as possible to a quarter section of the pot where the grass was cut at the soil level. We tried to avoid collecting from a previously sampled sector. Samples were immediately frozen in liquid nitrogen and lyophilized. Dried samples were ground to a powder in a Cyclotech 1093 mill and stored for analysis.

Alkaloid Extraction. A method described by Hill et al. (1993) with some modifications was used to extract alkaloids. Briefly, 100 mg of plant material were weighed and transferred into a liquid scintillation vial. One hundred nanograms of ergotamine were added as an internal standard. Nine ml of $CHCl_3$ and 1 ml of 0.01 N NaOH were added, and the sample was then agitated by wrist action for 30 min. The sample was filtered through Whatman 1 PS filter paper and loaded onto 5 ml solid-phase extraction columns prepared as described by Hill et al. (1993). The columns contained 0.5 g of Ergosil at the bottom and 1 g of Na_2SO_4 on top separated by Whatman No. 41 filter paper disks. Pigments were eluted with an aspirated manifold with 5 ml of acetone–chloroform (75:25), and the residual acetone–chloroform was removed with 1 ml of diethyl ether. A detachable Nylon 66 filter (22 μm) was attached to the syringe and the alkaloids eluted by slowly plunging 2 ml of methanol through. The sample was evaporated to dryness under N_2 flow and stored in the freezer untill analysis.

Separate extraction methods were attempted to see if they would be better suited for detection of peramine and lolitrem B in perennial ryegrass, as described by Ball et al. (1995). Only a few samples were subjected to these extractions. Chlorophylls remaining after the lolitrem extraction method were removed by passing the samples through an aminopropyl SPE column.

Data Collection. Samples were suspended in 100 μl of methanol, and analyzed on a Waters LC-MS system using a Novapak C_{18} (2 × 150 mm) column. The mobile phase was H_2O–methanol–acetonitrile plus 0.3% NH_4OH (60:10:20:10). The flow rate was 0.27 ml/min in a linear gradient for 48 min, changed to 10:10:70:10 at 52 min, and returned to the original composition untill 60 min. Peaks were detected with a Waters 996 Photodiode Array Detector, after which the flow was split in two with one part going through a Waters 474 Scanning Fluorescence Detector with excitation wavelength at 316 nm and emission

wavelength at 413 nm, and the other to the mass spectrometer. Splitting of the flow was done as a precautionary measure to avoid damage to the fluorometer cell by the pressure in the system. Positive ion electrospray spectra were recorded on a Waters ZQ instrument. Soft ionization of the electrospray results in production of quasimolecular ions $(M+H)^+$, where M represents molecular weight and H hydrogen.

Direct-phase chromatography of samples following Ball et al. (1995) was carried out as described by Gallagher et al. (1985) using a Supelco (2 × 150 mm) silica column and acetonitrile–CH_2Cl_2 (80:20) for 30 min. The excitation and emission wavelengths of the fluorescence detector were set at 265 nm and 440 nm, respectively.

Data Analysis. Quantification was based on the ergotamine internal standard. Repeated-measures (Statistica, StatSoft, Inc., Tulsa, Oklahoma, USA) analysis to look at the effect of time on alkaloid levels, and one-way ANOVA to compare samples from weekly and biweekly treatments at each sampling time were used. Replicates that could not be accurately determined due to interfering peaks in the chromatograms were excluded from analysis.

RESULTS

Alkaloids. Levels of major alkaloids and some of the unidentified putative alkaloids detected in tall fescue and perennial ryegrass are listed in Table 1. In tall fescue, we identified eight alkaloids, of which ergovaline, ergonovine, and ergocristine were the major ones. In addition, three unknown compounds were present in large quantities. In perennial ryegrass, ergocristine was the major alkaloid with two unidentified compounds. Evidence for eight more alkaloids was found using a method described by Ball et al. (1995).

Tall Fescue. The repeated-measures analysis of ergonovine indicated a significant effect by month (Figure. 1) ($F = 4.95$, $df = 3,12$, $P = 0.018$). Biweekly cutting resulted in higher levels of ergonovine ($F = 11.77$, $df = 1,3$, $P = 0.042$) by the second month.

The level of ergovaline in month 1 samples was higher in the grass subjected to weekly cutting than in grass clipped on a biweekly schedule ($F = 10.73$, $df = 1,3$, $P = 0.047$), but by month 2 the biweekly treatment showed a large increase over the weekly treatment ($F = 9.23$, $df = 1,4$, $P = 0.038$) (Figure 1). In the subsequent two months, a trend favoring biweekly mowing over weekly cutting was evident, but the differences were not significant due to large variation.

Like ergonovine, ergocristine levels showed an effect by month in the repeated-measures analysis ($F = 5.08$, $df = 3,12$, $P = 0.017$), but the cutting frequency was significant only at month 2 where weekly cutting gave higher values ($F = 6.81$, $df = 1,4$, $P = 0.059$). Levels remained high for the first three months and then decreased considerably in both treatments (Figure 1).

TABLE 1. ALKALOIDS AND PUTATIVE ALKALOIDS FOUND IN TALL FESCUE AND PERENNIAL RYEGRASS USING REVERSE-PHASE LC-MS ELECTROSPRAY IONIZATION[a]

Compound	$(M+H)^+$	Amounts (ng/g)
Tall fescue		
N-Formyl loline	183[b,c]	trace
N-Acetyl-loline	197	trace
Chanoclavine	257	<1
Ergonovine	326	71–490
Ergovaline	534	10–140
Ergocryptine	577	<1
Ergostine	596	trace
Ergocristine	611	84–312
Unknown A	537	370–4690
Unknown B	572	936–4196
Unknown C	574	48–391
Perennial ryegrass		
Peramine[b]	248	trace
Chanoclavine[b]	257	trace
Lysergamide[b]	268	trace
Lysergic acid[b]	269	trace
Ergonovine[b]	326	trace
Paxilline[b]	408	trace
Ergosine[b]	549	trace
Ergocristine	611	64–204
Lolitrem B[b]	686	trace
Unknown D	553	43–460
Unknown B	572	33–99

[a] The amounts (ng/g dry weight) are relative to an ergotamine internal standard. The compounds are in order of increasing size of their quasimolecular ions with known alkaloids listed first. $(M+H)^+$ denotes molecular weight (M) and hydrogen (H).

[b] Alkaloids found using the methods of Ball et al. (1995) and direct phase chromatography.

The three putative alkaloids (Figure 2, unknowns A, B, and C) all showed significant effects by month (A: $F = 63.02, df = 3,12; P = 0.001$; B: $F = 4.20, df = 3,12, P = 0.030$; C: $F = 3.66, df = 3,12, P = 0.044$), as well as month × treatment interactions (A: $F = 14.80, df = 3,12, P = 0.001$; B: $F = 8.34, df = 3,12, P = 0.003$; C: $F = 6.37, df = 3,12, P = 0.008$). During the second month of the study, all of the unknowns showed higher levels in samples from the biweekly cutting at month 2 (A: $F = 55.40, df = 1,4, P = 0.002$; B: $F = 103.27, df = 1,3, P = 0.002$; C: $F = 14.69, df = 1,4, P = 0.019$). Biweekly treatment also resulted in higher levels of unknowns A and B at month 4 (A: $F = 9.26, df = 1,4, P = 0.038$; B: $F = 9.59, df = 1,3, P = 0.053$).

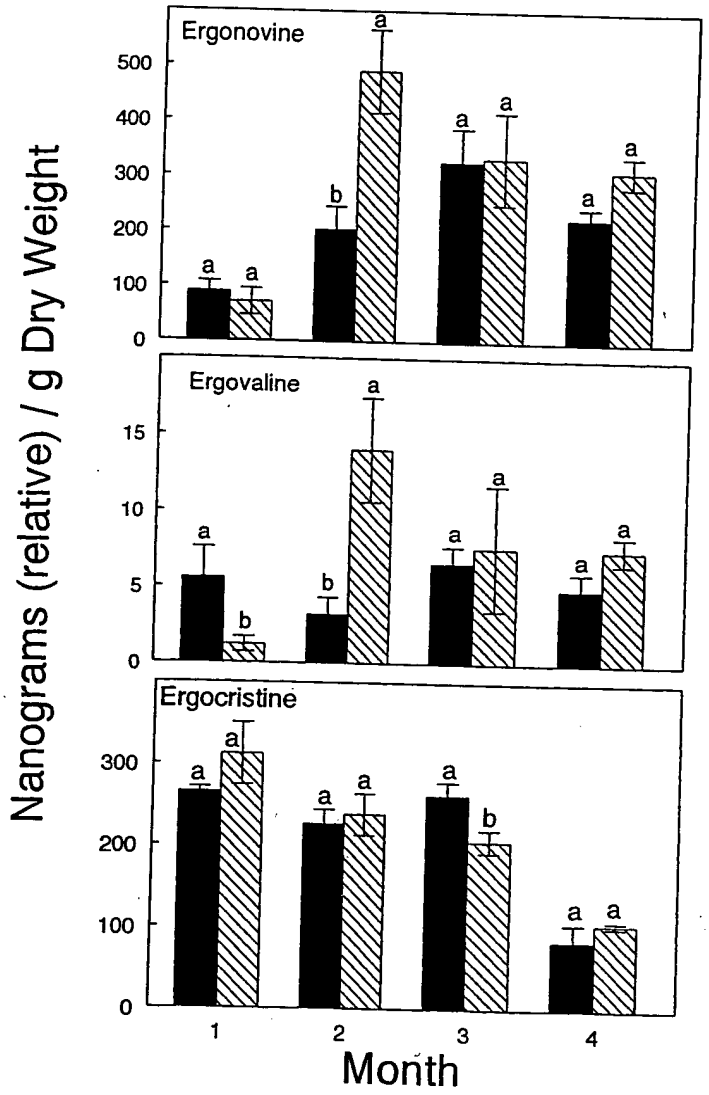
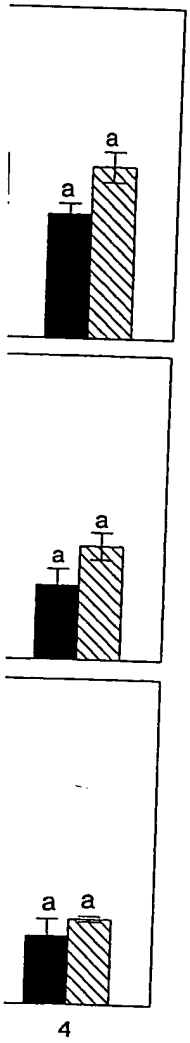


FIG. 1. Mean (±SE) levels of ergonovine, ergovaline, and ergocristine in tall fescue. Mowed weekly (■) or biweekly (▨). The same letters on the columns at each sampling date indicate no differences between treatments at $\alpha = 0.05$.



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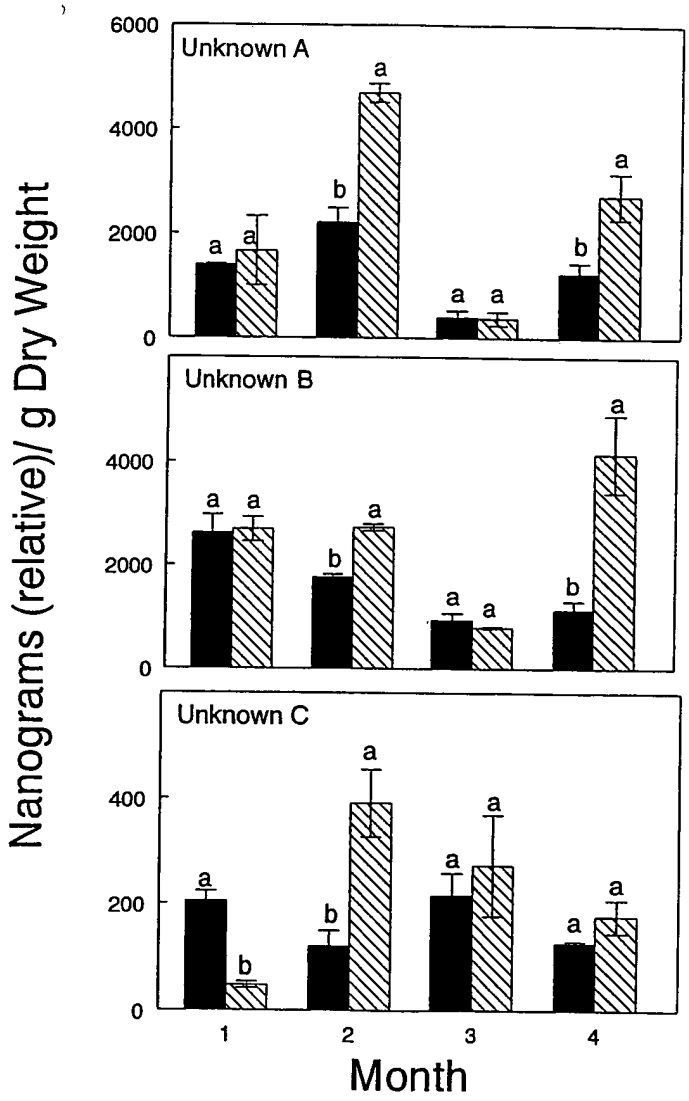


FIG. 2. Mean (±SE) levels of unknown A, unknown B, and unknown C in tall fescue. Mowed Weekly (■) or biweekly (▨). The same letters on the columns at each sampling date indicate no differences between treatments at $\alpha = 0.05$.

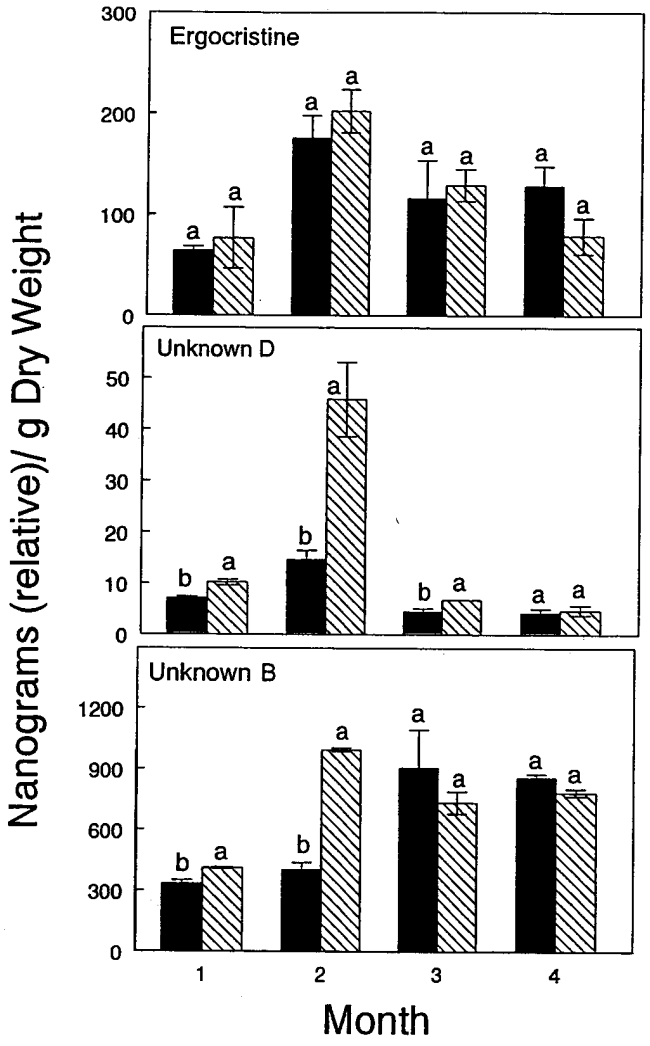
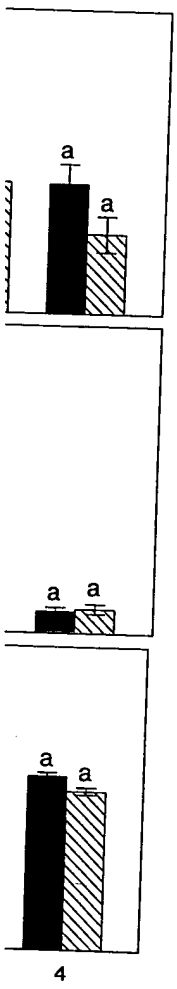


FIG. 3. Mean (±SE) levels of ergocristine, unknown D, and unknown B in perennial ryegrass. Mowed weekly (■) or biweekly (▨). The same letters on the columns at each sampling date indicate no differences between treatments at $\alpha = 0.05$.

Perennial Ryegrass. Only ergocristine was positively identified in the perennial ryegrass. Repeated-measures analysis indicated a main effect by month ($F = 8.17$, $df = 3,9$, $P = 0.006$), but no interaction. Biweekly treatments showed a slightly higher trend for the first three months over the weekly cutting (Figure 3). The level of ergocristine was similar to that in the tall fescue. For



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unknown D, the repeated-measures analysis indicated a significant effect by month ($F = 8.17$, $df = 3,9$, $P = 0.005$) and month × treatment interaction ($F = 14.00$, $df = 3,3$, $P = 0.028$) (Figure 3). Levels of unknown D were higher in the biweekly treatment for the first three months (month 1: $F = 28.77$, $df = 1,3$, $P = 0.013$; month 2: $F = 17.17$, $df = 1,4$, $P = 0.014$; month 3: $F = 16.04$, $df = 1,2$, $P = 0.057$) (Figure 3).

Samples from biweekly treatments contained more of unknown B than those from the weekly mowing for the first two months (month 1: ($F = 18.14$, $df = 1,2$, $P = 0.051$; month 2: $F = 292.90$, $df = 1,2$, $P = 0.003$). Unknown B is the same compound as unknown B in the tall fescue based on the quasimolecular ion 572, its fragmentation pattern, and retention time in the chromatograph. In addition, traces of peramine, chanoclavine, and ergostine were observed in some of the samples, but they were either poorly resolved or not detected in others (data not shown). The identity of other unknown peaks remains to be elucidated. The presence of several known alkaloids was observed in the few samples extracted by using the method of Ball et al. (1997) (Table 1).

In weekly mowing treatments of both grasses, we observed an oscillating variation in the alkaloid levels. No evidence for this was seen in the biweekly mowing treatment.

DISCUSSION

Our results agree with those reported by others on the general composition of endophytic alkaloids in tall fescue and perennial ryegrass [(Porter et al., 1979; Lyons et al., 1986; Siegel et al., 1990)]. Ergovaline is generally considered to be the predominant alkaloid in tall fescue (Lyons et al., 1986). It was somewhat surprising that the levels of ergovaline that we found were low in comparison to the other identified alkaloids. However, the levels vary greatly depending on the endophyte–host combination (Siegel et al., 1990; Breen, 1993). Values as high as 8 mg/g dry weight for loline alkaloids in tall fescue have been reported (Jones et al., 1983). Ergovaline levels from 166 to 1083 ng/g dry wt plant tissue have been shown in tall fescue (Rottinghaus et al., 1991).

A common trend across all samples was the increased level of alkaloids in tall fescue samples subjected to biweekly mowing. Although differences in individual as well as in putative alkaloids were small, taken together they indicate a more consistent pattern. Small increases could have an important impact on herbivore performance if there were interactions between the alkaloids. Yates et al. (1989) reported synergistic interaction between perloline and ergocryptine on mortality of *Oniopeltus fasciatus* (Dallas). In fact, most clear-cut differences were seen at month 2. The exception to the trend was at month 1 where the weekly treatments sometimes had a higher alkaloid content than plants treated biweekly.

Alkaloids are more concentrated in the sheaths than in leaves. Rottinghaus et al. (1991) reported ergovaline levels in tall fescue up to three times higher in leaf sheaths than leaves. Our samples were not separated into sheaths and leaves, and the grass was cut to the soil level. Samples from weekly cuttings would be expected to have a higher sheath-to-leaf ratio. Had the data indicated higher alkaloid content in samples from weekly cutting, this would have reflected the sheath-to-leaf ratio. As our data indicate a higher level in biweekly treatments, this would argue that the differences would be even more pronounced if the sheath-to-leaf ratio were the same in the two treatments. This is consistent with the reduction of alkaloid levels as a result of clipping when clipped and nonclipped tall fescue were compared (Belesky and Hill, 1997). Belesky and Hill (1997) suggest that carbon from photosynthesis is used to build new leaf material, and is, thus, not available for alkaloid synthesis. This could also contribute to the low levels of ergovaline found.

The unknown compounds exhibited trends similar to the identified alkaloids. However, we cannot be certain that these were, in fact, alkaloids. The extraction method and the solid-phase extraction, although yielding alkaloids, do not necessarily exclude other compounds with similar properties, e.g., phenolic compounds and chlorophylls. It should be pointed out that the unknown with a pseudomolecular weight of 572 is not a $(M+K)^+$ adduct of ergovaline, based on the absence of the $(M+H)^+$ spectral ion at 534.

The methods used did not provide good data for peramine or lolitrem B in the perennial ryegrass. Preliminary results using methods developed for peramine and lolitrem B extraction and direct-phase chromatography gave indications of the presence of not only these compounds, but also of ergosine, perloline methyl ether, paxilline, lysergic acid, and lysergamide in the perennial ryegrass samples. Lysergamide (ergine) may arise from solvolytic cleavage of lysergic acid during the extraction (Agurell, 1966). These data suggest that it may be necessary to analyze samples using different extraction and chromatography procedures to get a more comprehensive picture of the alkaloid profile in these grasses, e.g., ethanolic extraction of turf grasses has been successfully used for analysis of ergosterol (Logendra and Richardson, 1997).

Results with the perennial ryegrass must be interpreted with some caution because of heavy infestation by the aphid *Rhopalosiphum padi*. Several species of aphids show no avoidance of alkaloids (Latch et al., 1985; Siegel et al., 1990). Tall fescue has been shown to be resistant to *R. padi* (Johnson et al., 1985) and was not attacked, although it was growing next to the infested perennial ryegrass. A difference in the composition of the alkaloids in the two grasses could be one explanation for this. Our efforts to control the aphids with judicious application of azidirachtin were moderately successful, as we tried to avoid possible complications arising from the use of a synthetic pesticide.

It is tempting to speculate that if the period between cuttings had been extended to three weeks, the differences might have been more pronounced. From the

standpoint of a homeowner or a golf course manager, however, that might be impractical. In many of the weekly treatments, we noticed oscillating fluctuations in the alkaloid levels. Oscillations usually result from negative feedback (Goldbeter and Dupont, 1990) and could indicate that synthesis of alkaloids is regulated by changes in metabolism resulting from cutting, as resources are allocated toward building of new leaf material at the expense of secondary metabolites. Oscillations were not seen in the biweekly treatment.

The results do suggest the possibility that management practices, such as mowing frequency, could have an impact on alkaloid levels, and, as a consequence, on insect pest control in turf grasses. Specific endophyte–host genotypes providing control over insect pests are becoming available. Replacing established lawns and golf course fairways might not always be economically feasible. However, overseeding is a viable alternative, provided the new seed can establish itself in an existing stand (Richmond et al., 2000). The inconsistent control of insects with the use of endophytes (Clay et al., 1985) may have its basis not only in the endophyte–host combination, but also in cultural practices such as mowing frequency.

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SO Mikrobiologiya (1995), 64(5), 645-650

TI Alkaloids of Stipa robusta (sleepygrass) infected with an Acremonium endophyte
AU Petroski, Richard J.; Powell, Richard G.; Clay, Keith
CS Natl. Cent. Agric. Util. Res., Agric. Res. Serv., Peoria, IL, 61604, USA
SO Nat. Toxins (1992), 1(2), 84-8

TI Alkaloid composition of Penicillium palitans and Penicillium oxalicum
AU Vinokurova, N. G.; Reshetilova, T. A.; Adanin, V. M.; Kozlovskii, A. G.
CS Inst. Biochem. Physiol. Microorg., Pushchino, USSR
SO Prikl. Biokhim. Mikrobiol. (1991), 27(6), 850-5

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SO Prikl. Biokhim. Mikrobiol. (1990), 26(3), 355-9

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AU BACON C W; LYONS P C; PORTER J K; ROBBINS J D
CS TOXICOL. BIOL. CONSTIT. RES. UNIT, R.B. RUSSELL AGRIC. RES. CENT., USDA-ARS, ATHENS, GA.
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AU Cassady, John M.; Abou-Chaar, Charles I.; Floss, Heinz G.
CS Dep. Med. Chem. Pharmacogn., Purdue Univ., Lafayette, Indiana, USA
SO Lloydia (1973), 36(4), 390-6

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AU Erge, D.; Maier, W.; Groeger, D.
CS Inst. Biochem. Pflanzen, Halle/Saale, E. Ger.
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TI Production of alkaloids and related substances by fungi. III. Isolation of chanoclavine I and two new interconvertible alkaloids, regulovasine A and B, from Penicillium cultures
AU Abe, Matazo; Ohmomo, Sadahiro; Ohashi, Tsutomu; Tabuchi, Takeshi
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Institut für Biochemie der Pflanzen, Halle (Saale)
des Forschungszentrums für Molekularbiologie und Medizin
der Akademie der Wissenschaften der DDR

Untersuchungen über die enzymatische Umwandlung von Chanoclavin — I

Von D. Erge, W. Maier und D. Gröger

Mit 2 Abbildungen

(Eingegangen am 30. Oktober 1972)

Investigations on the Enzymatic Conversion of Chanoclavine — I

Summary

The properties of an enzyme system which catalyzes the ring closure of chanoclavine-I to agroclavine were investigated. The so called chanoclavine-I-cyclase is found only in saprophytically alkaloids producing strains of ergot. The timecourse of the appearance of chanoclavine-I-cyclase activity and the accumulation of alkaloids in submerged cultures of a clavine and of a peptide-type alkaloids producing strain was followed. The enzyme activity increased rapidly during the transition of the tropho-to the idiophase and decreases markedly after day 9—10 of fermentation. The reaction is strongly dependent on ATP and NAD^+ or $NADP^+$ but does not require FAD. Interestingly the conversion takes place also under anaerobic conditions. The crude enzyme extract could be stabilized by the addition of 30% glycerol to the buffer solution. The enzyme system converted chanoclavine-I, chanoclavine-I-aldehyd but not isochanoclavine-I to agroclavine. A slight feedback inhibition of the activity was caused by elymoclavine and lysergic acid. Preliminary results indicate that the chanoclavine-I-cyclase is an inducible enzyme system.

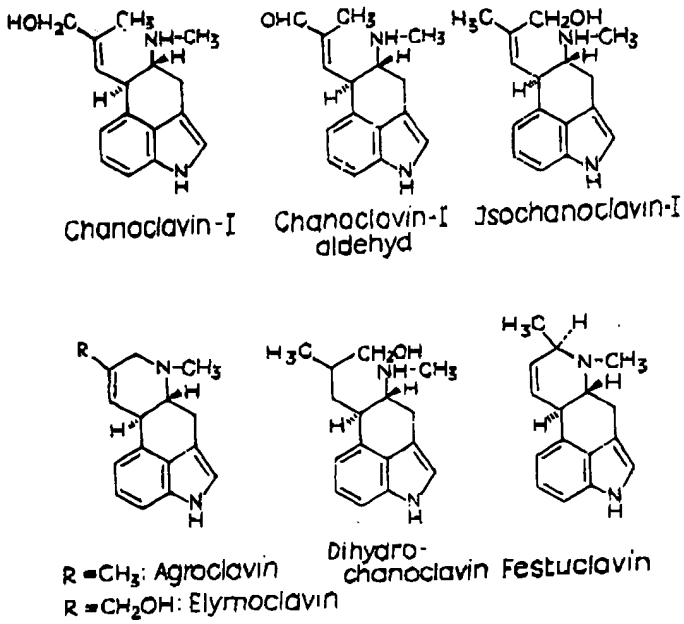
1. Einleitung

Für die Untersuchung biochemischer Regulationsprozesse sind eingehende Kenntnisse über die die Bildung der Metabolite katalysierenden Enzyme unbedingt erforderlich. Während im Bereich des Primärstoffwechsels ein umfangreiches Material vorliegt, sind bislang verhältnismäßig wenig für die Sekundärstoffbildung spezifische Enzyme nachgewiesen worden. Dies gilt auch für die Mutterkornalkaloide.

Es ist bekannt (15), daß in Claviceps-Arten folgende Biosynthesekette abläuft: Tryptophan und Mevalonsäure reagieren unter Bildung von Dimethylallyltryptophan. Das in 4-Stellung isoprenylierte Tryptophan kann via Chanoclavin-I → Agroclavin → Elymoclavin in Lysergsäure übergehen. Von der Lysergsäure leiten sich

einfache säureamidartige Derivate (z. B. Ergometrin) und die therapeutisch wichtigen Peptidalkaloide wie etwa Ergotamin ab.

Einige in vitro-Synthesen bzw. Alkaloidumwandlungen sind bislang bekannt geworden. Intensiv hat sich mit dieser Problematik ABE (1) beschäftigt. Die zellfreie Synthese verschiedener Clavine ist von CAVENDER und ANDERSON (6) beschrieben worden. Während im Rohextrakt des Claviceps-Stammes PRL 1980 keine Aktivität gefunden wurde, katalysierte die Fraktion „60–80% Ammonsulfat-Sättigung" die Umwandlung eines Gemisches von Isopentenylpyrophosphat, Methionin und Tryptophan in Chanoclavin, Agroclavin und Elymoclavin. Die gleiche Fraktion



Schema: Verschiedene Clavinalkaloide des Mutterkorns

war in der Lage Agroclavin zu Elymoclavin zu hydroxylieren (20). HEINSTEIN et al. (19) gelang als erste die Isolation der Dimethylallylpyrophosphat: L-Tryptophan-Dimethylallyltransferase aus dem Stamm SD 58. Die Bildung von Lysergyl-Co.A beschrieben (22). Nach OGUNLANA et al. (24) wandelt ein Rohenzym-Extrakt von Pennisetum-Mutterkorn (u. a. Stamm SD 58) Chanoclavin-I in Elymoclavin um, ohne daß dabei Agroclavin entstehen soll. Kürzlich wurde in unserem Laboratorium (16) der enzymatische Ringverschluß Chanoclavin-I→ Agroclavin gefunden. Über weitere Ergebnisse wird im folgenden berichtet.

2. Ergebnisse und Diskussion

2.1. Produktbildung

Unter unseren Versuchsbedingungen wurde Chanoclavin-I stets in das tetracyclische Agroclavin überführt. Elymoclavin und Setoclavin als mögliche Folgepro-

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dukte waren nach Inkubation mit dem Rohenzymextrakt nicht nachweisbar. Dieser Befund konnte durch Verwendung von Chanoclavin-I-^{14}C als Substrat, sowie die Charakterisierung des entstandenen Alkaloids durch DC in verschiedenen Lösungsmittelgemischen und massenspektrometrische Untersuchungen gesichert werden. Um quantitative Aussagen treffen zu können, wurde die Umwandlungsrate (% gebildetes Agroclavin) durch quantitative DC ermittelt. Wir möchten dieses Enzymsystem als Chanoclavin-I-Cyclase bezeichnen.

Hsu und Anderson (20), die ebenfalls einen Rohenzymextrakt eines Clavinbildners untersuchten, konnten keine Umwandlung von Chanoclavin-I feststellen. Bei den Versuchen von Ogunlana et al. (24), die den gleichen Stamm wie wir verwendeten, den Rohenzymextrakt aber auf andere Weise bereiteten, trat als einziges Umwandlungsprodukt Elymoclavin auf. Die mögliche Zwischenstufe Agroclavin konnten sie niemals nachweisen. Aus diesen Gründen postulieren sie einen direkten Weg von Chanoclavin-I → Elymoclavin. Im Verlauf dieser Reaktion soll die Doppelbindung $\Delta^{8,9}$ in Chanoclavin-I wandern und ein Epoxydring in 8.17-Position gebildet werden. Der endgültige Beweis für diese Annahme muß aber noch erbracht werden.

2.2. Fermentationsverlauf und Enzymaktivität

In Mutterkornstämmen, die saprophytisch keine Alkaloide bilden, konnten wir niemals Chanoclavin-I-Cyclase-Aktivität nachweisen. Kultiviert man Ergolinbildner unter Bedingungen, die keine Alkaloidsynthese erlauben, z. B. Claviceps paspali, in einem corn steep enthaltenden Medium, so ist dieses Enzymsystem ebenfalls nicht vorhanden. Alkaloidbildung und Cyclase-Aktivität sind offenbar streng korreliert, und

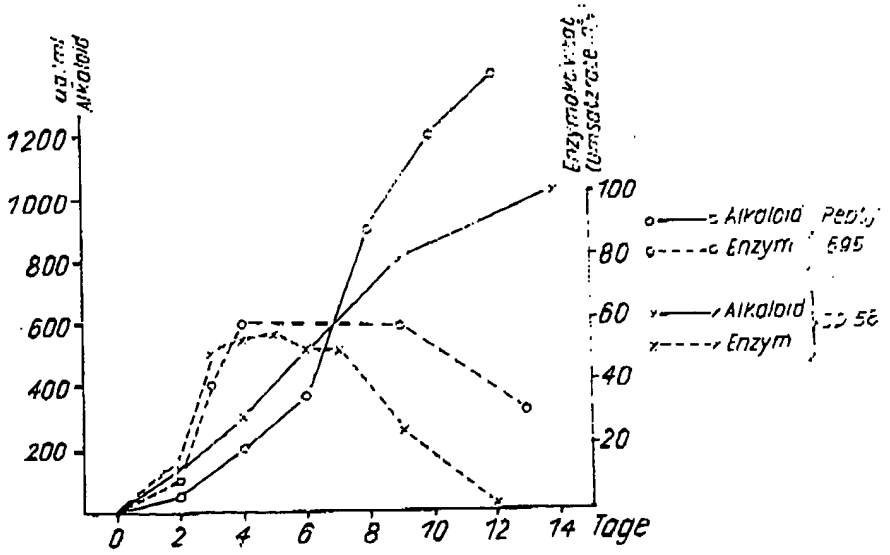

Abb. 1. Chanoclavin-I-Cyclase-Aktivität und -Alkaloidbildung im Laufe der Kulturentwicklung bei den Stämmen SD 58 und Pepty 695. (Enzymaktivität/0,5 g Frischmycel.) Fermentationsmedium NL 720.

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es war von Interesse, Alkaloidbildung und Enzymgehalt während des Fermentationsverlaufes zu untersuchen. Dazu dienten der Stamm SD 58 (Clavinbildner) sowie der Ergotoxinstamm Pepty 695 (Abb. 1). Beim Peptidbildner Pepty 695 ist die Wachstumsphase (bezogen auf den Proteingehalt) am 2. Tag abgeschlossen (7). Während der Trophophase ist nur eine geringe Enzymaktivität vorhanden. Ein rapider Zuwachs an Cyclase-Aktivität ist zwischen dem 2. bis 4. Kulturtag zu verzeichnen, also am Beginn der Idiophase. Das gleiche trifft zu für den Stamm SD 58. Der höchste Cyclasegehalt war beim Peptidbildner zwischen dem 4. bis 9. Kulturtag (Umsatzrate $\sim 60\,\%$) und beim Clavinproduzenten zwischen dem 3. bis 8. Kulturtag (Umsatzrate $\sim 50\,\%$) zu verzeichnen. Danach ist in beiden Stämmen ein Abfall des Enzymgehaltes zu beobachten. Am Ende der Fermentation ist beim Stamm SD 58 kein Enzym mehr vorhanden (12. Kulturtag). Das Maximum der Cyclase-Aktivität wird in beiden Stämmen vor dem Gipfel der höchsten Alkaloidwerte erreicht. Da die Cyclase einen Prozeß am Anfang der Ergolinbildung katalysiert, erscheint dies verständlich. Die Chanoclavin-I-Cyclase sowie die im Flossschen Laboratorium (19) gefundene Dimethylallylpyrophosphat : L-Tryptophan-Dimethylallyltransferase zeigen praktisch das gleiche Verhalten im Verlauf der Fermentation. Auch bei der Transferase ist ein Anstieg vor der Alkaloidsynthese und später ein starker Abfall der Enzymaktivität zu beobachten. Die Agroclavin-Hydroxylase (6) wurde in 9 Tage alten Hauptkulturen nachgewiesen; die Beziehungen zur Alkaloidbildung wurden offenbar nicht untersucht.

2.3. Einfluß von Cofaktoren und Versuche zur Stabilisierung

Die Abhängigkeit der Umwandlung von Chanoclavin-I in Rohenzym-Präparaten des Stammes SD 58 ist in Abb. 2 zusammengestellt. Die Auswahl der einzelnen Cofaktoren erfolgte in Anlehnung an Ogunlana et al. (24). Um eine optimale Agroclavinbildung zu erreichen, ist ein Zusatz von ATP notwendig. Unter unseren Bedingungen sind 10 μMol ATP/Ansatz erforderlich. Die geringfügige Umwandlung ohne ATP-Gabe ist wahrscheinlich auf Spuren von ATP oder verwandten Verbindungen zurückzuführen, die im Rohenzym-Extrakt enthalten sind. In welcher Weise ATP am Ringschlußmechanismus beteiligt ist, ist noch ungeklärt. Ogunlana et al. (24) postulieren, daß die Hydroxymethylgruppe phosphoryliert wird und durch Abspaltung des Phosphatrestes ein Carbonium-Ion entsteht, welches mit dem Stickstoff-Atom reagiert.

Weiter ergab sich, daß in einem bestimmten Bereich die Reaktion der Proteinkonzentration (2—8 mg/Ansatz) proportional ist. Im allgemeinen enthielten unsere Ansätze 10—12 mg Protein. Gut Alkaloid bildende Kulturen des Stammes SD 58 waren in der Lage, 0,1 mg Chanoclavin-I vollständig umzusetzen. Die Inkubationen wurden im allgemeinen mit 0,5 mg Substrat durchgeführt. Die Umsatzrate schwankte bei dieser Konzentration zwischen 60—80 %. Unter optimalen Bedingungen ent-

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standen bei einer Inkubationszeit von 4 h 0,3—0,4 mg Agroclavin! Eine Verlängerung der Inkubationszeit führte bei den verschiedenen Stämmen nicht zu einer stärkeren Agroclavinbildung. Im Bereich zwischen 28—35 °C ließen sich keine Unterschiede der Enzymaktivität feststellen. Nicht einheitlich war das Bild bei Zugabe von Mg^{++}. Um optimale Agroclavinbildung zu erzielen, war beim Peptidbildner ein Zusatz von 20 μMol $MgCl_2 \cdot 6\,H_2O$ notwendig, während beim Clavinbildner diese Menge Mg^{++} eine 50%ige Hemmung der Chanoclavin-I-Cyclase-Aktivität bewirkte. In Gegenwart von FAD tritt keine Agroclavinbildung ein. Strikt abhängig ist die Reaktion von der Zugabe von Nicotinamidnucleotiden. Ohne Zusatz von Pyridinnucleotiden war kein

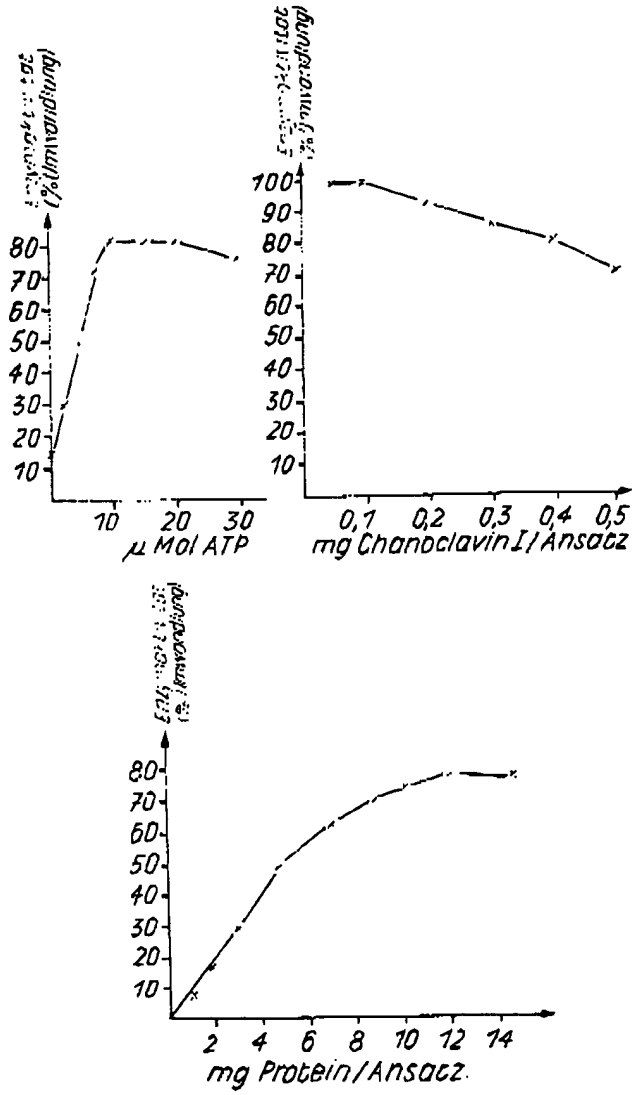

Abb. 2a. Abhängigkeit der Umwandlung von Chanoclavin-I von einzelnen Faktoren des Testansatzes. Allgemeiner Reaktionsansatz: siehe experimenteller Teil. Stamm SD 58. Fermentationsmedium NL 720. Alter des Mycels 5—6 Tage. Proteingehalt 12—14 mg/Ansatz. Mit Ausnahme der zu prüfenden Substanz waren alle anderen Faktoren in der Sättigungskonzentration vorhanden.

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Umsatz zu beobachten. Die Cyclase gehört offenbar zu den Enzymsystemen, bei denen beide Coenzyme wirksam sind, da mit NAD^+ sowie $NADP^+$ der Ringverschluß erfolgte. Höhere Umwandlungsraten ließen sich mit $NADP^+$ bzw. NADPH im Vergleich zu NAD^+ bzw. NADH erzielen. Noch ungeklärt ist, ob die oxydierte oder die reduzierte Form der Coenzyme primär in die Reaktion eingreift, da über den Mechanismus des Ringschlusses nichts bekannt ist. Falls der erste Schritt in der Bildung des Chanoclavinaldehyds besteht (23), könnte man annehmen, daß $NADPH^+$ oder NAD^+ als Coenzyme der entsprechenden Dehydrogenase fungieren. Die Tatsache, daß auch die jeweiligen reduzierten Formen der Pyridinnucleotide wirksam sind, erklärt sich zwanglos durch die Verwendung von Rohextrakten, in denen offenbar Reaktionen ablaufen, die zu NAD^+ und $NADP^+$ führen.

Durch Schütteln der Inkubationsansätze wurde weder eine Erhöhung der Umsatzrate beobachtet, noch konnten andere Alkaloide nachgewiesen werden. Der Ringschluß zum tetracyclischen Agroclavin läuft im Gegenteil auch unter anaeroben Bedingungen ab. Inkubationsversuche unter N_2 bzw. Argon ergaben die gleichen Werte wie Kontrollexperimente unter Normalbedingungen! Diese Befunde sollten bei einer zukünftigen Diskussion des Ringschlußmechanismus: Chanoclavin-I → Agroclavin Beachtung finden. Die bisherigen Vorstellungen gehen davon aus, daß am C-8 unter Allylumlagerung eine Oxydation erfolgt. Nach Elimination der möglicherweise phosphorylierten Hydroxylgruppe und Verschiebung der Doppelbindung würde ein Carbonium-Ion am C-10 entstehen, das durch elektrophilen Angriff am C-5 den Ring schließen könnte (8, 26). Die Beteiligung oxydierter Zwischenstufen bei der Elymoclavinbildung ist auch von (25) diskutiert worden.

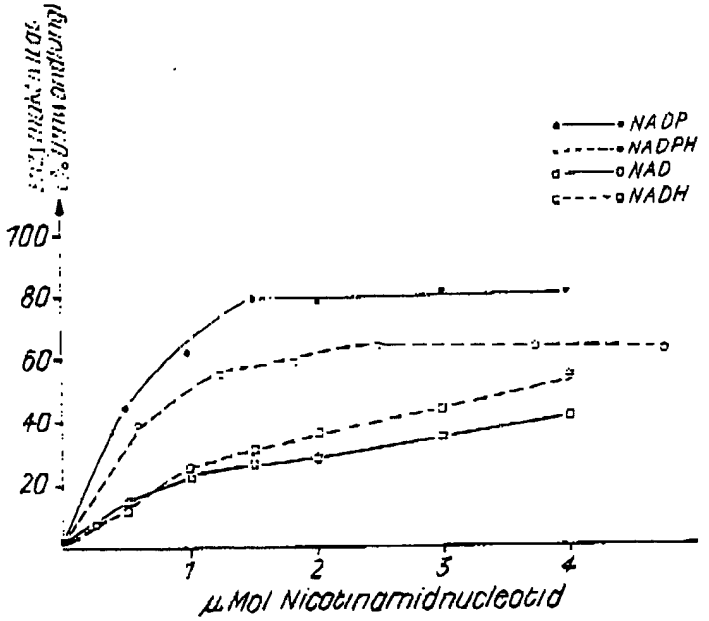

Abb. 2b. Legende wie Abb. 2a.

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Offenbar läuft aber die Verschiebung der Doppelbindung bzw. deren intermediäre Aufhebung (10) und der nachfolgende Ringschluß ohne Beteiligung oxydativer Prozesse ab.

Die Enzympräparate ließen sich ohne Aktivitätsverlust 7 Tage bei −40 °C aufbewahren. Eine Lagerung bei 0 °C führte schon nach 12 h zu völlig inaktiven Rohenzymextrakten. Eine bemerkenswerte Stabilisierung ließ sich durch einen 30%igen Zusatz von Glycerin zum Aufschlußpuffer erreichen. Unter diesen Bedingungen behielt der Rohenzymextrakt seine volle Aktivität bei 0 °C für 48 h.

Versuche zur Anreicherung des Enzymsystems sind bislang fehlgeschlagen. So ging der größte Teil der Aktivität durch Ammoniumsulfat-Fällung und anschließender Dialyse oder durch Chromatographie an einer Sephadex G-25-Säule verloren (Tabelle 1). Durch Zugabe verschiedener Extrakte konnte die Aktivität partiell wiederhergestellt werden. Dabei handelte es sich um Rohenzymextrakte, die selbst nicht in der Lage waren, die Umwandlung zu katalysieren (Zusatz A, D), oder um den nach Proteinfällung der Extrakte gewonnenen klaren Überstand (Zusatz C, D).

Tabelle 1. Chanoclavin-I-Cyclase-Aktivität im Rohenzymextrakt des Stammes SD 58 und nach Filtration über Sephadex G-25. Inkubationszeit: 4 h; Temperatur: 32 °C. Proteingehalt: 12–14 mg/Ansatz. Folgende Zusätze wurden appliziert: A: 0,5 ml Rohenzymextrakt SD 58 gewaschen in NL 611 1 d alt; B: 0,5 ml Überstand des Rohenzymextraktes SD 58 gewaschen in NL 611 nach Protein-Fällung (erhitzt 5 min unter N_2 auf 60 °C (20)); C: 0,5 ml Überstand des Rohenzymextraktes SD 58 gewachsen in NL 611 nach Proteinfällung durch Äthanol. D: 0,5 ml Rohenzymextrakt Li 342 gewachsen in einem corn steep medium

Nr.	Ansatz	Zusatz	µg[1]) Agroclavin	µg[1]) Chanoclavin	% Agroclavin	% Agroclavin ø
1	Rohenzymextrakt	—	120	56	68,2	68,9
			135	60	69,5	
2	Sephadex G-25-Filtrat	—	21	160	11,6	11
			19	164	10,3	
3	Sephadex G-25-Filtrat	A	40,7	141	22,4	23,1
			44,1	142	23,7	
4	Sephadex G-25-Filtrat	B	46,6	123	27,6	27
			46,6	129	26,5	
5	Sephadex G-25-Filtrat	C	39	100	28,1	27,6
			38	102	27,1	
6	Sephadex G-25-Filtrat	D	55	135	29	28,9
			42	122	28,8	
7	Fraktion 60–80% Ammonsulfat-Sättigung	—	24,5	103,6	19,1	18,7
			24,5	108,3	18,4	

1) nach DC-Trennung des Inkubationsansatzes

Diese Befunde machen es wahrscheinlich, daß neben den Pyridinnucleotiden und ATP noch weitere unbekannte niedermolekulare Cofaktoren an der Reaktion beteiligt sind. Ohne Erfolg wurden als Zusätze der Extrakte nach Chromatographie über Sephadex G-25 getestet: Vitamin Free Yeast Base, Liver Extract, Pyridoxalphosphat, Glutathion, Folsäure, verschiedene Pterine, CoA.

2.4. Substratspezifität und Wirkung von Effektoren

Von den tricyclischen hydroxylierten Clavinalkaloiden, den „Chanoclavinen", sind bislang vier verschiedene Stereoisomere bekannt geworden: Chanoclavin-I, Isochanoclavin-I, (—) Chanoclavin-II sowie das entsprechende Racemat. Lange Zeit war die Stellung der im Ring D geöffneten Clavine im Stoffwechsel des Mutterkorns unbekannt. Der eindeutige Einbau von Chanoclavin-I in tetracyclische Clavinalkaloide ist von GRÖGER et al. (17) beschrieben worden. Betrachtet man die stereochemischen Verhältnisse, so könnte man vermuten, daß Isochanoclavin-I, bei dem das Stickstoffatom der Hydroxymethylgruppe benachbart ist, als unmittelbare Vorstufe der tetracyclischen Ergoline fungiert. FEHR et al. (9) konnten als Erste aber eindeutig nachweisen, daß Isochanoclavin-I kein Vorläufer der Ergoline ist, sondern nur Chanoclavin-I inkorporiert wird. Auf Grund der Radioaktivitätsverteilung muß man annehmen, daß beim Übergang Chanoclavin-I → Agroclavin eine trans-cis Isomerisierung an der 8,9 Doppelbindung erfolgt. Zum gleichen Ergebnisse kamen FLOSS et al. (12, 13). Da FLOSS et al. (23) weiterhin zeigen konnten, daß Chanoclavin-I-aldehyd in Elymoclavin spezifisch eingebaut wird, kann man vermuten, daß der Aldehyd bei der Bildung des Agroclavins als essentielle Zwischenstufe durchlaufen wird. Durch VOIGT und ZIER (27) sind Dihydrochanoclavine zugänglich geworden, die in Spuren in Sklerotien sowie saprophytischen Kulturen bestimmter Stämme vorkommen. Nach (28) sollen die Dihydrochanoclavine in vivo in die entsprechenden Dihydroclavine sowie nach Dehydrierung in geringerem Maße in Agroclavin überführt werden.

Es war nun von Interesse zu untersuchen, welche Substrate durch unser Enzymsystem umgewandelt werden, d. h., es sollte geklärt werden, ob in vivo und in vitro die gleichen Reaktionen ablaufen. Zu diesem Zweck wurden die in Tabelle 2 angegebenen Alkaloide mit dem gleichen Rohenzymextrakt inkubiert. Wie zu erwarten, wurde auch bei diesem Versuch Chanoclavin-I in guten Ausbeuten (~ 60—65%) in Agroclavin überführt. Isochanoclavin-I dagegen wurde nicht umgewandelt, sondern wurde unverändert wiedergefunden. Praktisch die gleichen Verhältnisse liegen beim Dihydrochanoclavin vor. Die geringen nach DC-Trennung gefundenen Mengen an Agroclavin sind im Rohextrakt enthalten und sind nicht auf eine Umwandlung zurückzuführen. Da Festuclavin niemals im Rohenzymextrakt nachweisbar war, kann man schließen, daß unser Chanoclavin-I-Cyclase-Präparat in der Lage ist, Dihydrochanoclavin in Dihydroagroclavin umzuwandeln. Allerdings ist die Um-

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Tabelle 2 Substratspezifität der Chanoclavin-I-Cyclase. Stamm SD 58 gewachsen in NL 720. 20 mg Protein/Ansatz. Inkubationszeit: 4 h: Temperatur 32 °C

Nr.	Substrat	µg[1]			%	
		Agroclavin	Substrat[2]	Festuclavin	Agroclavin	Festuclavin
1	0,5 mg Chanoclavin-I	116	66	–	64	–
2	0,5 mg Chanoclavin-I	114	63	–	64,5	–
3	0,5 mg Chanoclavin-I	108	69	–	61	–
4	0,5 mg Chanoclavin-I	111	60	–	65	–
5	0,5 mg Isochanoclavin-I	~ 1	128	–	1–2	–
6	0,5 mg Isochanoclavin-I	~ 2	134			–
7	0,5 mg Isochanoclavin-I	~ 2	132	–		–
8	0,5 mg Isochanoclavin-I	~ 1	135	–		–
9	0,5 mg Dihydrochanoclavin	3	132	3	2,2	2,2
10	0,5 mg Dihydrochanoclavin	3	118	4	2,2	3,2
11	0,5 mg Dihydrochanoclavin	3	130	5	2,2	3,6
12	0,5 mg Dihydrochanoclavin	3	135	5	2,1	3,5
13	0,33 mg Chanoclavin-I-aldehyd[3]	45				
14	0,33 mg Chanoclavin-I-aldehyd[3]	46	–	–	46	
15	0,33 mg Chanoclavin-I-aldehyd[3]	48	–	–	48	
16	0,33 mg Chanoclavin-I-aldehyd[3]	50	–	–	50	
17	0,33 mg Chanoclavin-I-aldehyd-NADPH-ATP	14	–	–	15	
18		16	–	–	16	

1) Nach DC-Trennung des Inkubationsansatzes
2) 1–4: Chanoclavin-I, 5–8 Isochanoclavin-I, 9–12: Dihydrochanoclavin
3) berechnet auf 0,3 ml Alkaloidextrakt = 100 µg Chanoaldehyd

wandlungsrate wesentlich geringer als beim Einsatz von Chanoclavin-I. Wie aus den Versuchen 13–16 der Tabelle 2 hervorgeht, katalysiert die Cyclase auch den Ringschluß des Chanoclavin-I-aldehyds. Bei der Auftrennung der Reaktionsprodukte nach der Inkubation ließ sich der Aldehyd nur in Spuren nachweisen. Dies erklärt sich aus der Tatsache, daß diese empfindliche Substanz bei unserer Aufarbeitungsmethode (Ausschütteln des alkalisierten Inkubationsansatzes mit Chloroform) weitgehend zerstört wird. In Kontrollexperimenten ohne Enzymzusatz konnte niemals eine Agroclavinbildung nachgewiesen werden. Nach Inkubation von Chanoclavin-I-aldehyd-U-^{14}C ließ sich radioaktives Agroclavin isolieren, welches die gleiche spezifische Radioaktivität wie das Substrat aufwies. Somit kann der enzymkatalysierte Ringschluß von Chanoclavin-I-aldehyd zu Agroclavin als gesichert gelten. Wir haben zahlreiche Versuche mit Chanoclavin-I-U-^{14}C (kurze Inkubationszeiten sowie „Ab-

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fangen" mit Semicarbazid) durchgeführt, um Chanoclavin-I-aldehyd nachzuweisen. Dies ist uns in keinem Fall gelungen. Offenbar wird der möglicherweise als Intermediärprodukt auftretende Chanoclavin-I-aldehyd sofort an der Enzymoberfläche weiter umgesetzt und läßt sich nicht anreichern. Zusammenfassend ergibt sich auch aus den in vitro-Versuchen, daß Chanoclavin-I als Schlüsselverbindung in der Biosynthesekette DMA-Tryptophan → Chanoclavin-I → tetracyclische Clavine anzusehen ist und kein anderes Chanoclavin-Isomeres eine obligatorische Zwischenstufe ist.

In weiteren Versuchen wurde der Einfluß von Effektoren auf die Chanoclavin-I-Cyclase-Aktivität geprüft. Zum Inkubationsansatz wurden die Effektoren in verschiedenen Konzentrationen ($2 \cdot 10^{-4}$ M, $1 \cdot 10^{-3}$ M) hinzugefügt. Getestet wurden Tryptophan bzw. einige Methylderivate dieser Aminosäure sowie Elymoclavin und Lysergsäure. L- und D-Tryptophan sowie D,L-5- und D,L-6-Methyltryptophan beeinflußten unter diesen Bedingungen die Aktivität der Cyclase praktisch nicht. Lediglich D,L-4-Methyltryptophan zeigte in einer Konzentration von $1 \cdot 10^{-3}$ M eine geringe Hemmwirkung. Besonders bei *C. fusiformis* SD 58 (4, 10, 11) ist wiederholt gezeigt worden, daß die Ergolinbildung ein induzierbarer Prozeß ist. Tryptophan bzw. verschiedene Methylderivate davon sind hier besonders wirksam. Der Induktionseffekt ist am größten bei Zugabe der Induktoren in der Trophophase. Bei Claviceps paspali hemmt D,L-4-Methyltryptophan die Bildung einfacher Lysergsäurederivate (2). Die biochemischen Grundlagen des Induktionseffektes sind bislang ungeklärt. Nach (4) soll ein „metabolisch labiles" Enzym induziert werden. Ganz sicherlich handelt es sich bei der von Bu'Lock und Barr (4) diskutierten „ergoline synthetase" nicht um ein einzelnes Enzym, sondern um einen ganzen Enzym-Komplex. Aktivitätsänderungen der Chanoclavin-I-Cyclase durch potentielle Induktoren der Ergolinsynthese ließen sich unter unseren Bedingungen jedenfalls nicht beobachten.

Offenbar unterliegt die Dimethylallylpyrophosphat: Tryptophan-Dimethylallyltransferase der Feedback Regulation. Agroclavin und Elymoclavin sollen die Transferase hemmen (19). Über den Umfang der Hemmung und die Konzentrationen der Effektoren ist nichts bekannt (19). Elymoclavin und Lysergsäure — Ergoline, die in der Biosynthesekette hinter Agroclavin liegen — verursachen in einer Konzentration von $1 \cdot 10^{-3}$ M eine 15—20%ige Hemmung der Chanoclavin-I-Cyclase-Aktivität (Tabelle 3). Somit ergeben sich gewisse Parallelen zur Transferase, welche die Bildung des DMA-Tryptophans katalysiert. Beide Enzyme stehen am Anfang der Ergolinbildung und vermindern ihre Aktivität, wenn die Endprodukte sich in hohen Konzentrationen in den Zellen anreichern und könnten so als einer der limitierenden Faktoren der Alkaloidsynthese am Ende der Idiophase angesehen werden.

Wir haben erste Hinweise, daß die Chanoclavin-I-Cyclase induzierbar ist und es sich nicht bei diesem Stamm um ein konstitutives Enzym handelt wie etwa die 6-Methylsalicylsäuresynthetase in Penicillium urticae (5).

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Tabelle 3 Der Einfluß von Effektoren auf die Chanoclavin-I-Cyclase-Aktivität im Rohenzym extrakt des Stammes SD 58. Kultivation des Pilzes in NL 720. Inkubationszeit: 4 h Temperatur 32 °C. Proteingehalt 14 mg/Ansatz

Nr.[1])	Effektor		μg[2]) Agroclavin	μg[2]) Chanoclavin	% Agroclavin
1	—		81	24	77,1
2	L-Tryptophan	$2 \cdot 10^{-4}$ M	76	28	73
3	L-Tryptophan	$1 \cdot 10^{-3}$ M	69	27	71
4	D-Tryptophan	$2 \cdot 10^{-4}$ M	79	32	71,2
5	D-Tryptophan	$1 \cdot 10^{-3}$ M	74	28	72,5
6	D,L-4-Methyltryptophan	$2 \cdot 10^{-4}$ M	80	19	81
7	D,L-4-Methyltryptophan	$1 \cdot 10^{-3}$ M	78	37	67,7
8	D,L-5-Methyltryptophan	$2 \cdot 10^{-4}$ M	85	32	72,5
9	D,L-5-Methyltryptophan	$1 \cdot 10^{-3}$ M	97	20	83
10	D,L-6-Methyltryptophan	$2 \cdot 10^{-4}$ M	88	30	73,5
11	D,L-6-Methyltryptophan	$1 \cdot 10^{-3}$ M	86	31	73,5
12	Elymoclavin	$2 \cdot 10^{-4}$ M	71	33	68,8
13	Elymoclavin	$1 \cdot 10^{-3}$ M	65	50	56,5
14	Lysergsäure	$2 \cdot 10^{-4}$ M	76	32	70,4
15	Lysergsäure	$1 \cdot 10^{-3}$ M	71	43	62

1) Mittelwerte aus 2 Versuchen
2) nach DC-Trennung des Inkubationsansatzes

Kultiviert man den Clavinbildner in einem phosphatreichen Medium, so lassen sich weder Ergoline noch die Cyclase nachweisen. In Parallelexperimenten mit der gleichen Nährlösung (NL 611), aber mit dem Zusatz von Tryptophan oder D,L-5-Methyltryptophan, wurden Alkaloide gebildet und die Cyclase gefunden. Nach 5tägiger Kultivation betrug der Alkaloidgehalt 150 $\mu g/ml$ und die Umsatzrate des Enzyms 14–15%. In der Standardnährlösung NL 720 waren beim gleichen Versuch 380 $\mu g/ml$ Alkaloid nachweisbar und die Umsatzrate betrug 56%.

Mit der weiteren Klärung des Mechanismus der Umwandlung Chanoclavin-I → Agroclavin sowie Versuchen zur Anreicherung der Chanoclavin-I-Cyclase sind wir zur Zeit beschäftigt.

Material und Methoden

1. Mutterkornstämme:

a) Claviceps purpurea-Stamm Pepty 695. Unter saprophytischen Bedingungen werden Clavine, Ergometrin und Ergotoxin-Alkaloide gebildet.

b) Verschiedene Claviceps purpurea-Stämme, die parasitisch Ergotamin bilden, bei saprophytischer Kultur aber keine Ergoline synthetisieren.

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c) Claviceps fusiformis, Stamm SD 58 (Pennisetum-Mutterkorn). Neben Elymoclavin und Agroclavin als Hauptalkaloide werden Penniclavin, Chanoclavin und in Spuren unbekannte Ergoline saprophytisch gebildet.

d) Clavicepspas pali-Stamm Li 342. Als Hauptalkaloid liegt saprophytisch Chanoclavin-I neben den isomeren Lysergsäuremethylcarbinolamiden vor.

2. Nährlösungen:

NL 611: 50 g Mannit; 50 g Saccharose; 5,4 g Bernsteinsäure; Hefeextrakt Difco 3,0 g; KH_2PO_4 1,0 g; $MgSO_4 \cdot 7\,H_2O$ 0,3 g; $FeSO_4 \cdot 7\,H_2O$ 10 mg; $ZnSO_4 \cdot 7\,H_2O$ 4,4 mg Aqua dest. ad 1000,0. *p*H eingestellt mit konz. Ammoniak auf *p*H 5,4.

NL 614: Wie NL 611 an Stelle von 1 g KH_2PO_4 nur 0,1 g KH_2PO_4.

NL 619: Sorbit 50,0 g; Ammoniumsuccinat 30,0 g; 1,2-Propandiol 20 ml; KH_2PO_4 1,0 g; $MgSO_4 \cdot 7\,H_2O$ 0,3 g; Aqua dest. ad 1000,0. *p*H eingestellt auf 5,4.

NL 720: Saccharose 200—300,0 g; Ammoncitrat 15,0 g; KH_2PO_4 0,25 g; $MgSO_4 \cdot 7\,H_2O$ 0,3 g; $FeSO_4 \cdot 7\,H_2O$ 10 mg; $ZnSO_4 \cdot 7\,H_2O$ 30,8 mg. Aqua dest. ad 1000,0; *p*H 5,2—5,4.

3. Kultivation:

Zur Anzucht des Impfmaterials der einzelnen Stämme wurde Mycel von Agarröhrchen bzw. Oberflächenkulturen verwendet. Die Vorkulturen wurden 8 Tage geschüttelt und danach in die Hauptkultur übertragen. Als Kulturgefäße dienten 500 ml Langhalskolben. Die Kultivation erfolgte auf Rundschüttelmaschinen des VEB Fanal, Bad Frankenhausen, bei einer Temperatur von 24 °C ± 1 °C.

Als Vorkulturmedien wurden verwendet: NL 611 für SD 58; ein corn steep solids enthaltendes Medium für C. paspali sowie NL 720 + 0,2 g/L Difco Hefeextrakt für C. purpurea.

4. Präparate:

NAD, NADH, NADP, NADPH: VEB Arzneimittelwerk Dresden sowie Sigma, St. Louis; Glutathion red., Mercaptoäthanol: Ferrak Berlin; Coenzym A, Folsäure, FAD, Pyridoxalphosphat: Boehringer, Mannheim; Vitamin Free Yeast Base, Yeast Extract: Difco, Detroit; Tris: Merck, Darmstadt; Sephadex G-25, Pharmacia, Uppsala.

Dihydrochanoclavin (Gemisch von 80% Dihydrochanoclavin-I und 20% Isodihydrochanoclavin-I) von Prof. Dr. R. Vater, Berlin. Chanoclavin-I wurde aus der Nährlösung von Claviceps-paspali-Kulturen isoliert. Schmp. 215 °C (Boetius-Heiztisch). $[\alpha]_D^{20} = -244°$ Pyridin (14). Chanoclavin-I-[^{14}C] haben wir nach Applikation von Na-acetat-2-^{14}C an C. paspali-Kulturen (Fütterungsdauer 6 Tage) gewonnen. Isochanoclavin-I wurde durch Bestrahlung von Chanoclavin-I, gelöst in einem Gemisch von tert. Butanol/Benzol (6:1), mittels einer Quecksilber-Niederdrucklampe NK 6/20 Hanau 254 nm erhalten (8). Die Isolation erfolgte durch wiederholte Dünnschichtchromatographie an Kieselgel G „Merck". Schmp. 188 °C. DC: $R_F = 0{,}24$ (Chloroform/Methanol 95:5 gesättigt mit Ammoniak). Chanoclavin-I-aldehyd (23): In Aceton gelöstes Chanoclavin-I haben wir mit MnO_2 versetzt und das Ganze 45 Min. am Rückfluß erhitzt. Durch präparative DC (Chloroform/Methanol (8:2)) ließ sich in 50%iger Ausbeute der Aldehyd gewinnen. $R_F \sim$: 0,5—0,6. MS (Atlas CH-6 bei 70 eV): m/e 254 (M^+); m/e 154 (typisch für tri- und tetracyclische Ergoline (3)).

Beim Ansprühen mit van Urk's Reagenz reagierte die Verbindung unter Violettfärbung, die nach etwa 30 Min. in blau überging. Elymoclavin isolierten wir durch Säulenchromatographie an Aluminiumoxyd basisch Akt. I „Woelm" aus Submerskulturen des Stammes SD 58. Agroclavin: Koch & Light, Colnbrook; Festuclavin und Pyroclavin verdanken wir Prof. Abe, Tokyo.

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5. Gewinnung des Rohenzym-Extraktes:

Frisches, mit Wasser und Puffer (*p*H 7,4) gewaschenes Mycel wurde im Verhältnis 1:1 in 0,1 M Tris-HCl-Puffer *p*H 7,4 suspendiert (1 L Puffer enthielt 100 mg Chelapex II + 0,4 *ml* Mercaptoäthanol) und mit der X-Press (Bios, Schweden) aufgeschlossen. Der Aufschluß gelang auch durch inniges Verreiben des frischen Mycels mit Trockeneis. Die anschließende Zentrifugation erfolgte 60 Min. in der Ultrazentrifuge (VAC-60) (Janetzki) bei 50000 U/Min. Der Überstand wurde für die Untersuchungen eingesetzt. Alle Operationen bei 0—4 °C.

6. Enzymansatz:

Der Ansatz enthielt im Gesamtvolumen von 2 *ml* 10 μMol ATP; 1,25 μMol NADPH; 0,5 mg Chanoclavin-I; 200 μl 1 M Tris-HCl-Puffer (*p*H 7.4) und beim Stamm Pepty 695 20 μMol $MgCl_2 \cdot 6H_2O$. Im allgemeinen wurden 0,5 *ml* Rohenzym-Extrakt zugegeben. Inkubation bei 32 °C 4 h. Die Reaktion wurde durch kurzes Erhitzen abgestoppt. Die angegebenen Werte in den Abbildungen und Tabellen wurden durch zwei Inkubationsansätze ermittelt. Von jedem Ansatz hat man zur Bestimmung der Umsatzrate zwei DC-Trennungen durchgeführt.

7. Aufarbeitung und Analytik:

Nach Beendigung der Inkubation hat man den Ansatz mit Ammoniakflüssigkeit alkalisch gemacht (*p*H 10) und die Alkaloide mit Chloroform ausgeschüttelt. Die vereinigten Auszüge wurden vorsichtig eingeengt und auf 1 ml aufgefüllt. Von dieser Lösung hat man ~0,3 *ml* strichförmig auf DC-Platten aufgetragen (Kieselgel PF_{254} „Merck") und im Lösungsmittelgemisch Chloroform/Methanol (8:2) entwickelt. Zum qualitativen Nachweis wurden die Chromatogramme mit van Urk's Reagenz besprüht. Zur quantitativen Bestimmung wurden die unter UV-Licht angezeichneten Alkaloidzonen vorsichtig abgeschabt und in Zentrifugenröhrchen überführt. Danach wurden 2 *ml* einer Mischung von 45 *ml* Methanol: 45 *ml* Wasser: 10 *ml* Eisessig hinzugegeben und einige Min. vorsichtig umgeschüttelt. Nach Hinzufügen von 4 *ml* van Urk's Reagenz wurde erneut einige Min. geschüttelt und das Ganze anschließend zentrifugiert. Die klaren blaugefärbten Lösungen wurden 6 Min. im UV-Licht bestrahlt und die Extinktion im Elko II (d = 0,5) S 57 gemessen. Die Alkaloidwerte hat man Eichkurven entnommen, die unter gleichen Bedingungen aufgestellt wurden. Mit diesem Verfahren ließen sich ~125—140 μg Gesamtalkaloid (75–85 %) der eingesetzten Menge nach der DC-Trennung wiedergewinnen. Zur Ermittlung der Umsatzrate setzte man den nach der DC-Trennung gefundenen Gesamtalkaloidgehalt (bestehend aus Chanoclavin + Agroclavin) = 100 %.

Aliquote Teile der Nährlösung extrahierte man nach Alkalisieren erschöpfend mit Chloroform, dampfte ein und nahm den Rückstand mit 2%iger Weinsäure auf. Weitere Bestimmung nach (22). Die Proteinbestimmung erfolgte nach Lowry et al. (21). Zur Radioaktivitätsmessung diente ein Tricarb-Scintillationsspektrometer Modell 3365 der Fa. Packard. Als Scintillatorflüssigkeit verwendeten wir eine Lösung von 5 g POP und 0,3 g POPOP in 1000 *ml* Toluol p.a.

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TI Ergot alkaloid biosynthesis by isolates of Balansia epichloe and B. henningsiana
AU Bacon, Charles W.; Porter, James K.; Robbins, Joe D.
CS Richard B. Russell Res. Cent., USDA, Athens, GA, 30613, USA
SO Can. J. Bot. (1981), 59(12), 2534-8

TI Conformations of the ergot alkaloids chanoclavine-1, aurantioclavine, and N-acetylaurantioclavine [Claviceps purpurea, Fungi].
AU Sakharovskii, V.G.; Aripovskii, A.V.; Baru, M.B.; Kozlovskii, A.G.
AV DNAL (QD241.K453)
SO Chemistry of natural compounds., Sept/Oct 1983 (pub. 1984) Vol. 19, No. 5. p. 626-627

TI Peptide-type ergot alkaloids produced by Hypomyces aurantius
AU Yamatodani, Saburo; Yamamoto, Isao
CS Kobe Women's Junior Coll., Kobe, 650, Japan
SO Nippon Nogei Kagaku Kaishi (1983), 57(5), 453-6

Biosynthesis of ergot alkaloids. Mechanism of the conversion of chanoclavine-I into tetracyclic ergolines
AU Floss, Heinz G.; Tcheng-Lin, Marie; Chang, Ching-Jer; Naidoo, Bala; Blair, Garre E.; Abou-Chaar, Charles I.; Cassady, John M.
CS Dep. Med. Chem., Purdue Univ., West Lafayette, Indiana, USA
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AU Porter, James K.; Bacon, Charles W.; Robbins, Joe D.; Betowski, Don
CS Richard B. Russell Agric. Res. Cent., United States Dep. Agric., Athens, GA, USA
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TI ALKALOIDS FROM THE FUNGUS CLAVICEPS-SP IBPM-F-401.
AU KOZLOVSKII A G; ARINBASAROV M U; SOLOV'EVA T F; ADANIN V M; GRIGOROV I; ANGELOV T I; SLOKOSKA L S; ANGELOVA M B
CS INST. BIOCHEM. PHYSIOL. MICROORG., ACAD. SCI. USSR, PUSHCHINO, USSR.
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90:199927
TI Ergosine, ergosinine, and chanoclavine I from Epichloe typhina
AU Porter, James K.; Bacon, Charles W.; Robbins, Joe D.
CS Richard B. Russell Agric. Res. Cent., Sci. Educ. Adm., Athens, Ga., USA
SO J. Agric. Food Chem. (1979), 27(3), 595-8

I LABORATORY PRODUCTION OF ERGOT ALKALOIDS BY SPECIES OF BALANSIA.
AU BACON C W; PORTER J K; ROBBINS J D
CS US FIELD CROPS LAB., US SCI. EDUC. ADM., R. B. RUSSELL AGRIC. RES. CENT., ATHENS, GA. 30604, USA.

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ПРИКЛАДНАЯ БИОХИМИЯ И МИКРОБИОЛОГИЯ

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АЛКАЛОИДЫ ГРИБА *CLAVICEPS SP.* ИБФМ-F-401

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Из фильтрата культуральной жидкости гриба *Claviceps sp.* ИБФМ-F-401 выделены в индивидуальном виде 6 алкалоидов. Физико-химическими и химическими методами эти алкалоиды идентифицированы как элимоклавин, агроклавин, ханоклавин-I, сетоклавин, пенниклавин и изопенниклавин.

Исследования в области изучения биосинтеза эргоалкалоидов, поиска новых соединений этого класса ведутся в течение нескольких десятков лет [1—3]. Большой интерес был вызван широким спектром биологической активности эргоалкалоидов, в особенности алкалоидов ряда лизергиновой кислоты, классическими продуцентами которых являются грибы рода *Claviceps*, паразитирующие на различных видах растений. После разработки способов глубинного культивирования ряда штаммов грибов рода *Claviceps* они стали использоваться в практике для получения ценных фармакологических препаратов [4].

В последнее время особое внимание было обращено на клавиновые алкалоиды, которые, по современным данным, оказались перспективными для лечения целого ряда заболеваний. Такие алкалоиды, как элимоклавин, агроклавин, пенниклавин, наряду с обычным для ряда эргоалкалоидов действием на центральную нервную систему, гладкую мускулатуру матки являются ингибиторами пролактина и, по имеющимся данным, возможно, могут быть использованы для лечения рака молочной железы, заболеваний предстательной железы [5, 6].

Таким образом, поиск культур микроорганизмов — продуцентов эргоалкалоидов, установление строения компонентов образующейся смеси алкалоидов, изучение их биосинтеза являются актуальной задачей как с точки зрения практики, имея в виду использование этих соединений в медицине, так и с чисто теоретической точки зрения, в частности для выяснения деталей биосинтеза этих соединений, их роли в процессах обмена веществ у микроорганизмов.

Целью данной работы являлось установление строения компонентов суммарной смеси алкалоидов, продуцируемых грибом *Claviceps sp.* ИБФМ-F-401.

ЭКСПЕРИМЕНТАЛЬНАЯ ЧАСТЬ

Методика

В качестве продуцента алкалоидов использовали гриб *Claviceps sp.* ИБФМ-F-401. Колонии этого гриба на глюкозо-картофельном агаре на 24-й день культивирования достигали 50 *мм* в диаметре, белые, обратная сторона колоний пурпурного оттенка, пигмент такого же цвета диффундировал в агар. Растущий край ровный, поверхность колонии войлочная. Центральная часть колонии с возрастом приобретала бежево-пурпурный оттенок, становилась бархатистой.

Таблица 1

При культивировании на лабораторной среде (T_2), содержащей сахарозу 100 *г*, DL-аспарагин 5 *г*, $Ca(NO_3)_2 \cdot 4H_2O$ 1 *г*, KH_2PO_4 0,25 *г*, $MgSO_4 \cdot 7H_2O$ 0,25 *г*, дрожжевой экстракт 0,1 *г*, KCl 0,12 *г*, $FeSO_4 \cdot 7H_2O$ 0,015 *г*, агар-агар 20 *г*, воду водопроводную до 1 *л*, HCl до pH среды 5,2, штамм образовывал белый воздушный мицелий и давал конидиальное спороношение типа *Sphacelia*. Конидиеносцы палочковидные, простые, бесцветные, 11,2—14,4×3,2 *мк*. Конидии одноклеточные, бесцветные, эллипсоидальной формы, 4,8—8×3,2 *мк*.

Культуру *Claviceps sp.* поддерживали в пробирках со скошенной агаризованной средой T_2. Выращивание осуществляли в течение 12 дней при 24°. Полученную таким образом культуру высевали в колбы объемом 750 *мл* со 150 *мл* среды следующего состава: глюкоза 100 *г*, лимонная кислота 10 *г*, KH_2PO_4 0,5 *г*, $MgSO_4 \cdot 7H_2O$ 0,3 *г*, дрожжевой экстракт 0,1 *г*, $FeSO_4 \cdot 7H_2O$ 0,007 *г*, $ZnSO_4 \cdot 7H_2O$ 0,006 *г*, водопроводная вода до 1 *л*, значение pH среды доводили концентрированным аммиаком до 5,2, гриб выращивали в течение 6 дней на качалке 170—180 *об/мин* при температуре 24°. Полученную культуру использовали для инокуляции колб объемом 750 *мл*, содержащих 150 *мл* среды: сахароза 300 *г*, лимонная кислота 15 *г*, KH_2PO_4 0,5 *г*, $MgSO_4 \cdot 7H_2O$ 0,25 *г*, дрожжевой экстракт 0,1 *г*, KCl 0,12 *г*, $FeSO_4 \cdot 7H_2O$ 0,007 *г*, $ZnSO_4 \cdot 7H_2O$ 0,006 *г*, вода водопроводная до 1 *л*, значение pH среды доводили до 5,2 концентрированным аммиаком. Культивирование проводили на качалке при 24°.

Качественный и полуколичественный контроль за ходом накопления эргоалкалоидов в фильтрате культуральной жидкости осуществляли методом ТСХ на пластинках «Silufol» (с УФ-индикатором и без него) в системе хлороформ — метанол — конц. аммиак (90 : 10 : 1 *об/об*). Количественный метод основан на спектрофотометрическом определении суммарного содержания эргоалкалоидов при 283 *нм* (в пересчете на элимоклавин, М. В. 254). Пробы получали в результате трехкратной экстракции алкалоидов хлороформом (3 : 1 *об/об*) из культуральной жидкости при pH 7,5, хлороформенный экстракт упаривали в вакууме досуха, остаток растворяли в этиловом спирте и спектрофотометрировали.

Масс-спектры записывали на масс-спектрометре МС-1302 с использованием прямого ввода образца в источник ионов при ионизирующем напряжении 60 *эв* и при температуре испарения пробы 0—160°. Спектры ПМР снимали на приборе «Varian HA-100D» (США) при 34° для растворов в дейтеропиридине, внутренний стандарт — ТМС. УФ-спектры получены на приборе «Specord UV-VIS» (ГДР). ИК-спектры записывали на спектрофотометре UR-20 (таблетки KBr). Температуры плавления определены на нагревательном столике Кофлера. Углы оптического вращения снимали на приборе 141 М «Perkin Elmer» (Англия). Тонкослойная хроматография осуществлялась на пластинах «Silufol» UV-254 («Kavalier», ЧССР) и на незакрепленных слоях Al_2O_3 IV ст. активности («Реахим», ТУ 6-09-426-70). В качестве сорбентов для колоночной хроматографии использовались силикагель L 40/100 *мк* («Chemapol», ЧССР) и окись алюминия различной активности («Реахим», ТУ 6-09-426-70). Контроль за ходом элюции алкалоидов с колонок осуществляли методом ТСХ на соответствующих сорбентах в тех же растворителях. В качестве стандартных образцов использовали хроматографически чистые препараты элимоклавина, агроклавина, ханоклавина-I, сетоклавина, пенниклавина и изопенниклавина, полученные в данной работе. Стандартные образцы эргокриптина, эргокристина и тартрата эрготамина получены от фирмы «Sigma» (США), эргометрин фирмы «Fluka AG, Buchs SG» (Швейцария), эрготаминин фирмы «Merck» (Darmstadt).

Для выделения суммарной смеси алкалоидов культуральную жидкость фильтровали, фильтрат подщелачивали до pH 7,5 и многократно

Таблица 1

Физико-химические данные выделенных соединений

Вещество	Т. пл.	$[\alpha]_D^{20°}$ в пиридине	УФ-спектр, в этаноле, λ *нм* (lgε)
Элимоклавин	236—239°	—138° ($c = 0{,}93$) *	$\lambda_{макс}$ 226 (4,44) 277 пл. (3,80) 283 (3,82) 293 (3,75) $\lambda_{мин}$ 249 (3,34) 291 (3,82)
Лит. [14—16,18] данные	245—247°	—152° ($c = 0{,}9$)	$\lambda_{макс}$ 227 (4,31) 283 (3,84) 293 (3,76)
Агроклавин	202—205°	—144° ($c = 0{,}9$) *, **	$\lambda_{макс}$ 225 (4,48) 277 пл. (3,79) 283 (3,81) 293 (3,73) $\lambda_{мин}$ 247,5 (3,23)
Лит. [14,15,18] данные	205—206°	—155° ($c = 0{,}9$) **	$\lambda_{макс}$ 225 (4,47) 284 (3,88) 293 (3,81)
Ханоклавин-I	214—216,5°	—214° ($c = 0{,}56$)	$\lambda_{макс}$ 224 (4,51) 273 пл. (3,82) 283 (3,84) 292 (3,78) $\lambda_{мин}$ 245,5 (3,21) 290 (3,77)
Лит. [14,16—18] данные	220—222°	—207° ($c = 0{,}5$)	$\lambda_{макс}$ 225 (4,44) 284 (3,82) 293 (3,76) $\lambda_{макс}$ 275 пл. (3,86) 281 (3,89) 291 (3,82) $\lambda_{мин}$ 245, 289
Сетоклавин	225—229°	+230° ($c = 1{,}0$) ***	$\lambda_{макс}$ 242,5 (4,40) 313 (4,02)
Лит. [14,18] данные	229—234°	+232° ($c = 1{,}1$) ***	$\lambda_{макс}$ 243 (4,38) 313 (4,04)
Пенниклавин	203—209°	+162° ($c = 0{,}52$) *	$\lambda_{макс}$ 242; 314, $\lambda_{мин}$ 236; 270
Лит. [15,14,18] данные	222°	+151° ($c = 0{,}5$)	$\lambda_{макс}$ 240 (4,29) 315 (3,93)
Изопенниклавин	163—165°	+150° ($c = 0{,}69$)	$\lambda_{макс}$ 242 (4,31) 313 (3,94)
Лит. [15,14] данные			

* $t = 24°$; ** в хлороформе; *** снято на полосе 5461 Å.

Таблица 1

Физико-химические данные выделенных соединений

Вещество	Т. пл.	$[\alpha]_D^{20°}$ в пиридине	УФ-спектр, в этаноле, λ *нм* (lgε)
Элимоклавин	236—239°	—138° ($c = 0,93$) *	$\lambda_{макс}$ 226 (4,44) 277 пл. (3,80) 283 (3,82) 293 (3,75) $\lambda_{мин}$ 249 (3,34) 291 (3,82)
Лит. [14—16,18] данные	245—247°	—152° ($c = 0,9$)	$\lambda_{макс}$ 227 (4,31) 283 (3,84) 293 (3,76)
Агроклавин	202—205°	—144° ($c = 0,9$) *, **	$\lambda_{макс}$ 225 (4,48) 277 пл. (3,79) 283 (3,81) 293 (3,73) $\lambda_{мин}$ 247,5 (3,23)
Лит. [14,15,18] данные	205—206°	—155° ($c = 0,9$) **	$\lambda_{макс}$ 225 (4,47) 284 (3,88) 293 (3,81)
Ханоклавин-I	214—216,5°	—214° ($c = 0,56$)	$\lambda_{макс}$ 224 (4,51) 273 пл. (3,82) 283 (3,84) 292 (3,78) $\lambda_{мин}$ 245,5 (3,21) 290 (3,77)
Лит. [14,16—18] данные	220—222°	—207° ($c = 0,5$)	$\lambda_{макс}$ 225 (4,44) 284 (3,82) 293 (3,76) $\lambda_{макс}$ 275 пл. (3,86) 281 (3,89) 291 (3,82) $\lambda_{мин}$ 245, 289
Сетоклавин	225—229°	+230° ($c = 1,0$) ***	$\lambda_{макс}$ 242,5 (4,40) 313 (4,02)
Лит. [14,18] данные	229—234°	+232° ($c = 1,1$) ***	$\lambda_{макс}$ 243 (4,38) 313 (4,04)
Пенниклавин	203—209°	+162° ($c = 0,52$) *	$\lambda_{макс}$ 242; 314, $\lambda_{мин}$ 236; 270
Лит. [15,14,18] данные	222°	+151° ($c = 0,5$)	$\lambda_{макс}$ 240 (4,29) 315 (3,93)
Изопенниклавин	163—165°	+150° ($c = 0,69$)	$\lambda_{макс}$ 242 (4,31) 313 (3,94)
Лит. [15,14] данные			

* $t = 24°$; ** в хлороформе; *** снято на полосе 5461 Å.

экстрагировали порциями (3 : 1 *об/об*) хлороформа. Контроль за полнотой экстракции осуществляли с помощью реактива Ван Урка [7]. Для ускорения разделения слоев использовали центрифугирование на центрифуге К-70 (20 мин. при 5000 *об/мин*). Объединенный хлороформенный экстракт высушивали над Na_2SO_4, упаривали в вакууме досуха. Полученную таким образом суммарную смесь алкалоидов использовали для дальнейшего исследования.

Для выделения элимоклавина использовали фильтрат из 3,3 *л* культуральной жидкости после 12 суток культивирования. По описанной выше методике было получено 2,79 *г* суммарной смеси алкалоидов. Полученную суммарную смесь обрабатывали 100 *мл* кипящего метанола, раствор охлаждали, выпавший осадок отфильтровывали и перекристаллизовывали из кипящего метанола (1 часть элимоклавина на 70 частей метанола). Элимоклавин (вес 1,74 *г*) кристаллизуется из метанола в виде призм, т. пл. 236—239° (с разложением). Вещество плохо растворимо в большинстве органических растворителей и воде, хорошо растворимо в пиридине, хуже—в метаноле.

Выделенные кристаллы анализировали для идентификации методами ИК-, УФ- и ПМР-спектроскопии, а также масс-спектрометрически.

Маточник после выделения элимоклавина (1,05 *г*) хроматографировали на колонке с окисью алюминия V ст. активности (вес сорбента 280 *г*, диаметр колонки 28 *мм*, элюент—хлороформ, скорость элюции 0,25 *мл/мин*, объем фракций по 5 *мл*). Контроль за ходом разделения осуществляли методом ТСХ. Каждую фракцию анализировали методом ТСХ на таком же сорбенте (подвижная фаза хлороформ). В качестве свидетеля использовали полученные ранее кристаллы, идентифицированные как элимоклавин. Фракции с 33-й по 80-ю, по данным ТСХ, содержали элимоклавин, ханоклавин-I и пенниклавин. После повторной хроматографии фракций, обогащенных элимоклавином, на колонках с силикагелем (элюент хлороформ — метанол — конц. аммиак в соотношении 90 : 10 : 1), было получено дополнительно 250 г элимоклавина.

Физико-химические характеристики и данные по хроматографической подвижности выделенного элимоклавина приведены в табл. 1, 2 и на рис. 1, 2.

Агроклавин. 1 *г* суммарной смеси алкалоидов, выделенной экстракцией из 2 *л* культуральной жидкости после 6-суточной ферментации, разделяли методом колоночной хроматографии на окиси алюминия III—IV ст. активности (вес сорбента 300 *г*, диаметр колонки 26 мм).

Таблица 2

Хроматографическое поведение выделенных алкалоидов и стандартных образцов

Вещество	Флуоресценция в УФ-свете	Окраска с реактивом Эрлиха	«Silufol»				Al_2O_3 Д	IV ст. активности Е
			А	Б	В	Г		
Элимоклавин	—	Синяя	0,12	0,42	0,56	0,18	0,17	0,45
Агроклавин	—	»	0,31	0,83	0,81	0,55	0,67	0,80
Ханоклавин-I	—	»	0,05	0,44	0,14	0,09	0,09	0,32
Сетоклавин	Голубая	Зеленая	0,16	0,70	0,78	0,43	0,39	0,66
Пенниклавин	»	»	0,09	0,27	0,55	0,17	0,06	0,24
Изопенниклавин	»	»	0,15	0,33	0,65	0,21	0,11	0,25
Тартрат эрготамина	+	Синяя	0,65	0,30	0,89	0,44	0,17	0,61
Эрготамин	+	»	0,80	0,80	0,95	0,72	0,44	0,71
Эргокриптин	+	»	0,75	0,75	0,94	0,66	0,47	0,73
Эргокристин	+	»	0,76	0,74	0,94	0,68	0,46	0,72
Эргометрин	+	»	0,17	0,14	0,60	0,15	0,07	0,27
Фестуклавин	—	»	0,14	0,84	0,72	0,52	0,64	0,77

Примечание. Системы А: хлороформ — метанол (4:1); Б: хлороформ—диэтиламин (9:1); В: хлороформ — метанол — конц. аммиак (80:20:0.2); Г: то же в соотношении (90:10:1); Д: хлороформ; Е: хлороформ — метанол (100:2).

Элюент — хлороформ, скорость элюции 0,45 *мл/мин*, объем фракций по 6—7 *мл*. Контроль за ходом разделения осуществляли методом ТСХ, как описано выше, при выделении элимоклавина.

Фракции 36—43, содержащие, по данным ТСХ, индивидуальный агроклавин, упаривали в вакууме на роторном испарителе. Получено 496,4 *мг* остатка с т. пл. 186—190° (с разложением). При кристаллизации 140 *мг* «сырого» образца из горячего этилацетата выделено 47 *мг* кристаллического (светло-желтые призмы) агроклавина, т. пл. 202—205° (с разложением). Вещество растворимо в спиртах, хлороформе, пиридине, хуже — в ацетоне, этилацетате, бензоле, эфире, плохо — в воде.

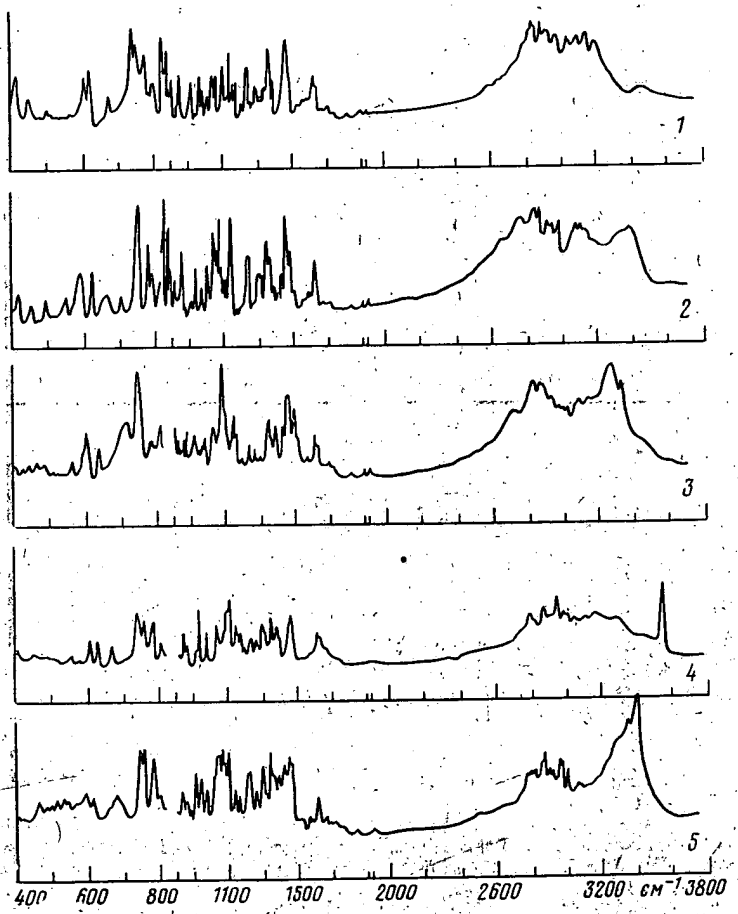

Рис. 1. ИК-спектры выделенных алкалоидов: *1* — агроклавин, *2* — элимоклавин, *3* — ханоклавин-I, *4* — сетоклавин, *5* — пенниклавин

Кроме того, получены фракции 44—50 (вес остатка 68 *мг*), обогащенные сетоклавином. Физико-химические характеристики и данные по хроматографической подвижности выделенного агроклавина приведены в табл. 1, 2 и на рис. 1, 2.

Ханоклавин-I. Фракция алкалоидов 33—45 (вес остатка 213 *мг*), состоящая в основном из элимоклавина и ханоклавина-I, полученная при хроматографировании маточника суммарной смеси алкалоидов после выделения элимоклавина на колонке с окисью алюминия, была далее разделена на отдельные компоненты на колонке с силикагелем (вес сорбента 70 *г*, диаметр колонки 22 *мм*). Элюент хлороформ—метанол—конц. аммиак в соотношении 90 : 10 : 1, скорость элюции

0,4 *мл/мин*, объем фракций по 4 *мл*. Фракции 150—156, содержащие ханоклавин-I упаривали в вакууме на роторном испарителе досуха. Всего получено 40 *мг* ханоклавина-I, т. пл. 214—216,5° (метанол, призмы). Вещество растворимо в метаноле, хуже в ацетоне, плохо в хлороформе, эфире, практически нерастворимо в гексане.

Физико-химические характеристики и данные по хроматографической подвижности выделенного ханоклавина-I приведены в табл. 1, 2 и на рис. 1, 2.

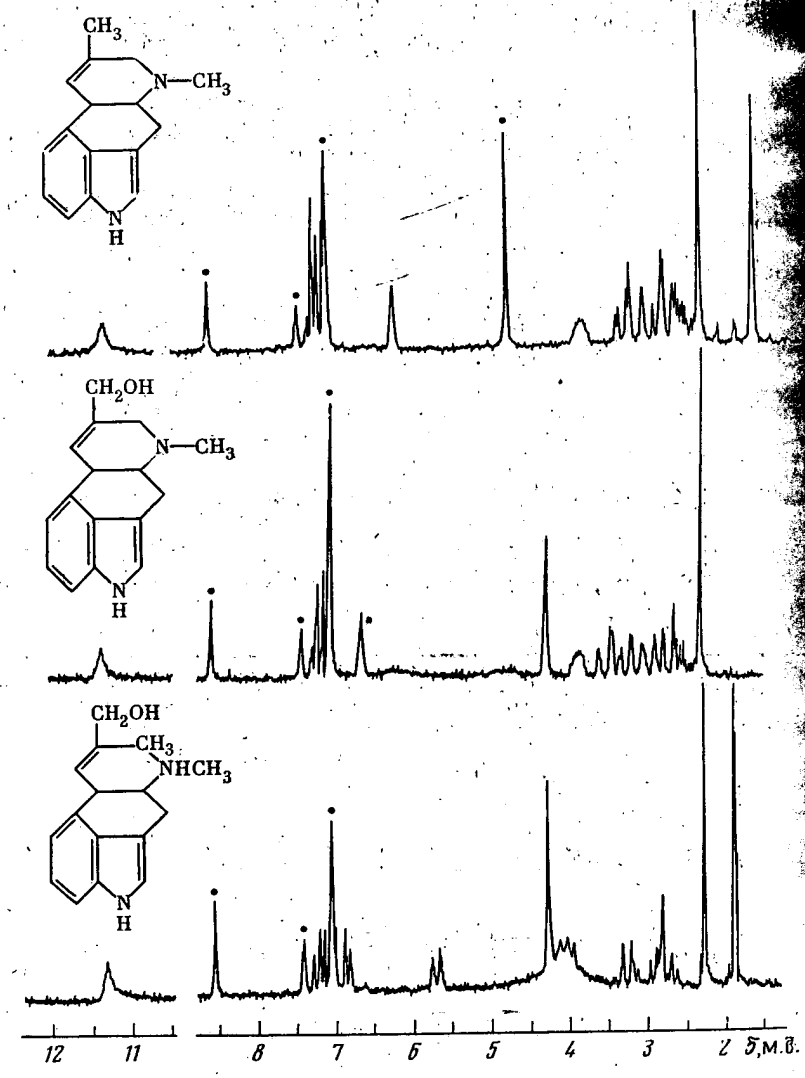

Рис. 2. ПМР-спектры агроклавина, элимоклавина, ханоклавина-I, * — наложившиеся сигналы растворителя

Сетоклавин. Часть фракции, 44—50 алкалоидов, обогащенная сетоклавином, при выделении агроклавина и содержащая в качестве основной примеси агроклавин, была разделена с использованием препаративной ТСХ на пластинах «Silufol» (проявитель хлороформ—диэтиламин в соотношении 9 : 1). Вещество элюировали из зон с R_f=0,70 метанолом. Элюат упаривали в вакууме досуха. Сетоклавин хорошо растворим в большинстве органических растворителей, плохо — в гексане, петролейном эфире, кристаллизуется из ацетона, т. пл. 225—229° (с разложением).

Физико-химические характеристики и данные по хроматографической подвижности выделенного сетоклавина приведены в табл. 1, 2 и на рис. 1.

Пенниклавин. При повторной колоночной хроматографии на окиси алюминия V ст. активности (вес сорбента 80 *г*, диаметр колонки 20 *мм*, элюент—хлороформ, скорость элюции 0,25—0,3 *мл/мин*, объем фракций по 4 *мл*) фракций 60—71 (вес 112 *мг*), полученных при хроматографировании маточника элимоклавина и содержащих в основном элимоклавин и пенниклавин, из фракций 40—50 выделено 8 *мг* пенниклавина. После перекристаллизации из ацетона получено 6 *мг* кристаллов, т. пл. 203—209° (с разложением). Вещество хорошо растворимо в спирте, хуже в ацетоне, хлороформе, плохо в гексане.

Физико-химические характеристики и данные по хроматографической подвижности выделенного пенниклавина приведены в табл. 1, 2 и на рис. 1.

Изопенниклавин. Выделен по той же схеме, как и пенниклавин, конечная очистка проводилась методом препаративной ТСХ на пластинах «Silufol» (проявитель хлороформ — метанол — конц. аммиак в отношении 80 : 20 : 0,2). Изопенниклавин элюировали метанолом из зоны с R_f=0,65. Элюат упаривали в вакууме досуха. Получено около 10 *мкг* изопенниклавина. Вещество хорошо растворимо в спирте, ацетоне, хуже — в хлороформе и плохо растворимо в гексане.

Физико-химические характеристики и данные по хроматографической подвижности приведены в табл. 1, 2.

Результаты и их обсуждение

С использованием описанных выше методов были выделены в индивидуальном виде 6 алкалоидов.

Все выделенные соединения давали положительную реакцию на эргоалкалоиды с реактивом Ван Урка, являлись основаниями, экстрагирующимися из водных растворов в органические растворители при щелочных значениях pH. Одним из основных компонентов смеси являлось соединение, которое было идентифицировано как элимоклавин. Это вещество, как и элимоклавин, поглощало в УФ-свете, его хроматографическая подвижность в различных системах на окиси алюминия и на закрепленных слоях силикагеля (пластины «Silufol») (табл. 2) соответствовала имеющимся литературным данным для элимоклавина [8]. Масс-спектр этого соединения характеризовался интенсивным молекулярным пиком с *m/e* 254 и максимальным по интенсивности пиком фрагмента [M—H] с *m/e* 253, которые являются характеристическими для элимоклавина. В спектре имелись типичные для эргоалкалоидов малоинтенсивные фрагментные пики с *m/e* 237, 223, 207, 194, 192, 182, 180, 167, 154, а также соответствующие пики двухзарядных ионов [9—11]. ИК- и УФ-спектры этого соединения (рис. 2, табл. 1) практически совпадали с имеющимися в литературе спектрами элимоклавина [12—15]. ПМР-спектр соединения (рис. 1) совпадал с ИК-спектром элимоклавина [2]. Угол оптического вращения (табл. 1) в пределах точности измерения совпадал с углом оптического вращения элимоклавина [2, 15]. В соответствии с литературными данными находилась также точка плавления этого соединения [14—16].

Второе соединение было идентифицировано как агроклавин. Оно поглощало в УФ-свете, его хроматографическое поведение (табл. 2) соответствовало имеющимся в литературе данным [8]. В масс-спектре этого соединения имелся характерный для агроклавина интенсивный пик молекулярного иона с *m/e* 238 и максимальный по интенсивности пик, соответствующий фрагменту [M—H], обладающему высокой стабильностью благодаря образованию стабилизированного за счет эффекта сопряжения бензониевого иона. В масс-спектре имелись также характерные для

эргоалкалоидов пики с *m/e* 223, 222, 221, 207, 196, 180, 167 и 154 [9—11]. ПМР- и УФ-спектры этого соединения (рис. 2, табл. 1) практически совпадали с соответствующими данными для агроклавина [12—15]. ИК-спектр этого вещества (рис. 1) совпадал с ИК-спектром агроклавина [14]. Точка плавления и угол оптического вращения (табл. 1) соответствовали литературным данным [14—15].

Третье соединение было идентифицировано как ханоклавин-I. В настоящее время известно 3 стереоизомерных вещества типа ханоклавина—ханоклавин-I, ханоклавин-II и изоханоклавин, обладающих одинаковым молекулярным весом, практически одинаковыми УФ-спектрами, одинаковыми цветными реакциями с реактивами Ван Урка и Келлера, сходными химическими свойствами; выделен также оптически неактивный рацемат ханоклавина-II. Однако эти соединения существенно различаются по их ИК-, ПМР-спектрам, углам оптического вращения и точкам плавления и в определенных условиях по хроматографической подвижности [17]. Хроматографическая подвижность выделенного вещества (табл. 2) соответствовала хроматографической подвижности ханоклавина-I [8]. Как и ханоклавин-I, это вещество поглощало в УФ-свете. В масс-спектре выделенного нами соединения имелся максимальный по интенсивности пик молекулярного иона с *m/e* 256. Характеристическими фрагментами являются фрагменты с *m/e* 238, 237, 183, 168, 155, 108. В спектре имелись соответствующие двухзарядные ионы. В целом масс-спектр не отличался существенно от масс-спектра ханоклавина-I [9, 10]. УФ-спектр вещества (табл. 1) являлся характерным для ханоклавина [14, 16]. ПМР- и ИК-спектры соединения (рис. 1, 2) существенно отличались от соответствующих данных для изоханоклавина и ханоклавина-II и практически совпадали с ПМР- и ИК-спектрами ханоклавина-I [16—18]. Угол оптического вращения (табл. 1) в пределах точности измерений совпадал с углом оптического вращения ханоклавина-I [17—18]. Существенное различие наблюдается в точках плавления ханоклавина-I, ханоклавина-II и изоханоклавина-I (222, 174 и 181° соответственно). Точка плавления выделенного вещества (табл. 1) практически не отличалась от точки плавления ханоклавина-I [16—18].

Четвертое соединение было идентифицировано как сетоклавин. Это соединение давало характерное зеленое окрашивание с реактивом Ван Урка, флюоресцировало в УФ-свете голубым светом, хроматографическая подвижность его (табл. 2) соответствовала хроматографической подвижности сетоклавина [8]. Масс-спектр вещества аналогичен масс-спектру сетоклавина [10]. В масс-спектре образца имелись интенсивный пик молекулярного иона с *m/e* 254, пара интенсивных пиков с *m/e* 236 [M—18] и с *m/e* 235 [M-19], а также пики, соответствующие фрагментам с *m/e* 219, 211, 210, 196, 181, 168, 167 и 154. УФ-спектр соединения (табл. 1) практически совпадал с УФ-спектром сетоклавина [14]. Как видно из рис. 1, практически нет различия между ИК-спектром выделенного нами образца и ИК-спектром сетоклавина [18]. Угол оптического вращения и точка плавления выделенного нами вещества (табл. 1) не отличались существенно от литературных данных для сетоклавина [18].

Пятое соединение было идентифицировано как пенниклавин. Вещество давало характерное зеленое окрашивание с реактивом Ван Урка, флюоресцировало при УФ-облучении голубым светом, его хроматографическое поведение (табл. 2) соответствовало литературным данным для пенниклавина [8]. Масс-спектр образца характеризовался максимальным по интенсивности пиком молекулярного иона с *m/e* 270 и набором интенсивных фрагментов с *m/e* 235, 219, 208, 196, 181 и 154 аналогично [10]. УФ- и ИК-спектры вещества (табл. 1, рис. 1) практически совпадали с имеющимися в литературе спектрами пенниклавина [15, 17, 18]. Угол оптического вращения и точка плавления выделенного соединения (табл. 1) хорошо согласовывались с литературными данными [15, 18].

Идентификация шестого соединения была осуществлена на основании данных по хроматографическому поведению вещества и анализа масс-спектра. Вещество давало характерное зеленое окрашивание с реактивом Ван Урка, флюоресцировало при УФ-облучении голубым светом. Хроматографическое поведение соединения (табл. 2) практически не отличалось от описанного в литературе для изопенниклавина [8]. Масс-спектр образца, так же как и масс-спектр пенниклавина, характеризовался максимальным по интенсивности пиком молекулярного иона с *m/e* 270 и набором интенсивных фрагментов с *m/e* 209, 208, 196, 181, 167, 155 и 154.

Таким образом, установлено, что гриб *Claviceps sp.* ИБФМ-F-401 продуцирует алкалоиды: элимоклавин, агроклавин, ханоклавин-I, сетоклавин, пенниклавин и изопенниклавин.

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ALKALOIDS FROM THE FUNGUS *CLAVICEPS SP.* IBPM-F-401

A. G. KOZLOVSKY, M. U. ARINBASAROV, T. F. SOLOVYOVA, V. M. ADANIN, I. GRIGOROV, T. I. ANGELOV, L. S. SLOCOSKA, M. B. ANGELOVA

Institute of Biochemistry and Physiology of Microorganisms, USSR Academy of Sciences, Pushchino, Institute of Microbiology, Bulgarian Academy of Science, Sofia

From the culture fluid filtrate of the fungus *Claviceps sp.* IBPM-F-401 six alkaloids were isolated. By physico-chemical and chemical methods these ergot alkaloids were identified to be elimoclavin, agroclavin, chanoclavin-I, setoclavin, penniclavin, and isopenniclavin.

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TI Alkaloids from the fungus Penicillium aurantio-virens Biourge and some aspects of their formation
AU Solov'eva, T. F.; Kuvichkina, T. N.; Baskunov, B. P.; Kozlovskii, A. G.
CS Inst. of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushkino, 142292, Russia
SO Mikrobiologiya (1995), 64(5), 645-650

TI Alkaloids of Stipa robusta (sleepygrass) infected with an Acremonium endophyte
AU Petroski, Richard J.; Powell, Richard G.; Clay, Keith
CS Natl. Cent. Agric. Util. Res., Agric. Res. Serv., Peoria, IL, 61604, USA
SO Nat. Toxins (1992), 1(2), 84-8

TI Alkaloid composition of Penicillium palitans and Penicillium oxalicum
AU Vinokurova, N. G.; Reshetilova, T. A.; Adanin, V. M.; Kozlovskii, A. G.
CS Inst. Biochem. Physiol. Microorg., Pushchino, USSR
SO Prikl. Biokhim. Mikrobiol. (1991), 27(6), 850-5

TI Enzymic transformation of chanoclavine-I by Penicillium sizovae F-209 cell-free extract
AU Markelova, N. Yu.; Kozlovskii, A. G.
CS Inst. Biochem. Physiol. Microorg., Pushchnio, USSR
SO Prikl. Biokhim. Mikrobiol. (1990), 26(3), 355-9

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ERGOT TOXICITY FROM ENDOPHYTE-INFECTED GRASSES A REVIEW.
AU BACON C W; LYONS P C; PORTER J K; ROBBINS J D
CS TOXICOL. BIOL. CONSTIT. RES. UNIT, R.B. RUSSELL AGRIC. RES. CENT., USDA-ARS, ATHENS, GA.
SO AGRON J, (1986) 78 (1), 106-116.

Ergot alkaloids. Isolation of N-demethylchanoclavine-II from Claviceps strain SD 58 and the role of demethylchanoclavines in ergoline biosynthesis
AU Cassady, John M.; Abou-Chaar, Charles I.; Floss, Heinz G.
CS Dep. Med. Chem. Pharmacogn., Purdue Univ., Lafayette, Indiana, USA
SO Lloydia (1973), 36(4), 390-6

WAM

Enzymic conversion of chanoclavine I
AU Erge, D.; Maier, W.; Groeger, D.
CS Inst. Biochem. Pflanzen, Halle/Saale, E. Ger.
SO Biochem. Physiol. Pflanz. (1973), 164(3), 234-47

TI Production of alkaloids and related substances by fungi. III. Isolation of chanoclavine I and two new interconvertible alkaloids, regulovasine A and B, from Penicillium cultures
AU Abe, Matazo; Ohmomo, Sadahiro; Ohashi, Tsutomu; Tabuchi, Takeshi
CS Tokyo Univ. Educ., Tokyo, Japan
SO Nippon Nogei Kagaku Kaishi (1969), 43(8), 575-82
CODEN: NNKKAA
DT Journal

Ergot alkaloids from plants

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第 43 巻 第 8 号 (昭和 44 年 8 月)

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〔農化 第 43 巻, 第 8 号, p. 575～582, 1969〕

菌類によるアルカロイドおよび関連物質の生産 (第3報)

Penicillium concavo-rugulosum の培養から chanoclavine-(I) および2種の新インドール・アルカロイド rugulovasine A および B の分離

阿部又三, 大桃定洋, 大橋 力, 田淵武士
(東京教育大学農学部)

昭和 44 年 3 月 5 日 受 理

Production of Alkaloids and Related Substances by Fungi
Part III. Isolation of Chanoclavine-(I) and Two New Interconvertible Alkaloids, Rugulovasine A and B, from the Cultures of *Penicillium concavo-rugulosum*

By Matazo ABE, Sadahiro OHMOMO, Tsutomu ŌHASHI and Takeshi TABUCHI
Faculty of Agriculture, Tokyo University of Education

Three Penicillium strains having the excellent ability to produce indole alkaloids in surface and submerged cultures were isolated from the air of the circumferance of laboratory. These strains were all identified as *Penicillium concavo-rugulosum* ABE.

By selection of the strains and modifying the medium, the total alkaloid yield in surface cultures could be increased up to *ca.* 1000 mg alkaloids per 10 cultures (culture filtrate 1.35 liter, and the mycelium 15 g, based on dry weight).

From these cultures, a hitherto known ergot alkaloid chanoclavine-(I) and, further, two new interconvertible alkaloids, rugulovasine A and B, have been isolated in good yields.

Rugulovasine A crystallized from benzene as colorless prisms ; $C_{16}H_{16}O_2N_2$, mp 138°C (decomp.), $[\alpha]_D^{22}$ 0°, $[\alpha]_{546}^{22}$ 0° ($c=0.5$, pyridine). Rugulovasine B separated from benzene as colorless resinous oil ; $C_{16}H_{16}O_2N_2$, $[\alpha]_D^{22}$ 0°, $[\alpha]_{546}^{22}$ 0° ($c=0.5$, pyridine). It yielded crystalline salts : the hydrochloride, $C_{16}H_{16}O_2N_2 \cdot HCl \cdot H_2O$, white prisms from water, mp 187°C (decomp.), $[\alpha]_D^{22}$ 0° ($c=0.5$, pyridine) ; the oxalate, $C_{16}H_{16}O_2N_2 \cdot 1/2\ C_2H_2O_4$, colorless prisms from water, mp 217°C (decomp.), $[\alpha]_D^{22}$ 0° ($c=0.5$, pyridine).

Both rugulovasine A and B gave reddish purple color, turning into bluish purple color with Allport and Cocking's reagent. They were reversibly converted into each other in various organic solvents, especially in alcohols. They were considered to be the chanoclavine type of new ergot alkaloid isomers, containing an opened D ring and an unsaturated lactone ring in their molecules.

The rugulovasines have been found with chanoclavine-(I) also in the cultures of various molds, such as *Penicillium rugulosum*, *Corticium caeruleum*, *Pellicularia filamentosa*, etc., besides *Penicillium concavo-rugulosum*. (Received March 5, 1969)

緒 言

著者ら[1]はさきに多種多様の既知糸状菌株について, それぞれの培養物中にいわゆる麦角アルカロイドが存在するかどうかを呈色反応とペーパークロマトグラフィーとによってしらべ, 分類学上の各項にまたがる多数の菌株に, 従来知られているクラビン型麦角アルカロイドのいずれかと一致する1つまたは2つ以上を含むいくつかのインドール・アルカロイド (インドール反応陽性塩基性物質) を生産する性質のあることを見出した.

さて, これら一連のアルカロイド, とくにペーパーク

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ロマトグラフィー上の挙動からはどの種の既知麦角アルカロイドとも同定できない不明の塩基性物質は，それぞれどのような物質であろうか．これを明らかにするためには，個々の物質を単離する必要があるが，しかしさきに検索されたどの種の菌株においても，それらはきわめて微量にしか生産されず，その目的を達成するには多大の困難が感じられる．この場合，天然からなんらかのインドール反応陽性塩基性物質をある程度まで多量に生産しうる糸状菌株をできるだけ多く探し出し，それらの生産する物質を追究するというやり方も，問題を堺り下げるための一つの方法であると考えられる．

こうした考えから，著者らは今回，まず目的にかなった菌株の探索につとめた．この場合，土壌，空気などから多数の糸状菌株を分離するとともに，各菌株を同じ条件の下で斜面培養し，その培養物の塩基性物質部分についてインドール反応陽性物質が含まれているかどうかを呈色反応でしらべ，反応の明瞭に認められたものについては薄層クロマトグラフィーによってその成分をしらべた．

その結果，実験室周辺の空気中から分離したペニシリウム属の3つの菌株の斜面培養で，少なくとも3種の麦角アルカロイド様塩基性物質“X”，“Y”および“Z”がこの順に著量生産されている事実が見出され，同時にそれら3菌株がいずれも *Penicillium concavo-rugulosum* ABE であることも知られた．そこで著者らは取りあえず，これら3菌株のうちから優良株1株を選択するとともにその培養条件をも設定して多量培養を実施し，その培養物について“X”，“Y”および“Z”の分離を試みた．その結果，まず“Z”を，ついで“X”をともに遊離の塩基として，さらに“Y”を塩酸塩などとしてそれぞれ結晶状に単離することに成功した．

ここに得た3種の物質のうち，“Z”はベンゼン，酢酸エチルなどから無色の柱状結晶として析出し，mp 220℃ (decomp.)，$[\alpha]_D^{22}$ −234°，$[\alpha]_{546}^{22}$ −286° (c=0.5, pyridine) を示し，分子式 $C_{16}H_{20}ON_2$ に相当した．これらの諸性質から，本物質が麦角アルカロイド chanoclavine-(I)[3] (secaclavine[3], chanoclavine[4]) であることには疑問の余地のないところであったが，その赤外線吸収スペクトルも chanoclavine-(I) のそれと全く一致した．つぎに“X”はベンゼンから無色の針状結晶として析出し，mp 138℃ (decomp.)，$[\alpha]_D^{22}$ 0°，$[\alpha]_{546}^{22}$ 0° (c=0.5, pyridine) を示し，分子式 $C_{16}H_{16}O_2N_2$ に相当した．本物質の塩酸塩は水から無色の柱状結晶として析出し，mp 225℃ (decomp.)，$[\alpha]_D^{22}$ 0° (c=1.0, pyridine)，組成 $C_{16}H_{16}O_2N_2 \cdot HCl$ を示した．またその修酸塩は水から無色の針状結晶として析出し，mp 224℃ (decomp.)，$[\alpha]_D^{22}$ 0° (c=1.0, pyridine)，組成 $C_{16}H_{16}O_2N_2 \cdot C_2H_2O_4$ を示したが，これに対して“Y”の塩酸塩は水から特徴ある白色の柱状結晶として析出し，mp 187℃ (decomp.)，$[\alpha]_D^{22}$ 0° (c=1.0, pyridine)，組成 $C_{16}H_{16}O_2N_2 \cdot HCl \cdot H_2O$ を示し，その修酸塩は水から無色の柱状結晶として析出し，mp 217℃ (decomp.)，$[\alpha]_D^{22}$ 0°(c=1.0, pyridine)，組成 $C_{16}H_{16}O_2N_2 \cdot 1/2\, C_2H_2O_4$ を示した．これらの諸性質から“X”と“Y”とは同じ分子式をもつ，相異なる新物質であると判断された．同時に，“X”および“Y”は相互に異性化しうること，ともに動物（猫）に対して特徴ある血圧降下作用を示すアルカロイドであることなども見出されたので，これらにそれぞれ rugulovasine A および B の名を与えた．rugulovasine A および B はその諸性質，とくにその赤外線吸収スペクトルから，ともに chanoclavine-(I) と同様，エルゴリン核の D 環が開裂した三環性麦角アルカロイドで，分子中に不飽和ラクトン環1個を有しているものと推定された．なお，両異性体はここに検索された *Penicillium concavo-rugulosum* 株に限らず，ほかの種々の糸状菌株によっても，常に chanoclavine-(I) を含む麦角アルカロイドまたは麦角アルカロイド様物質とともに生産されることが認められたが，これらの間の生成上の関係については目下検討中である．

実験ならびに結果

I. インドール反応陽性塩基性物質生産性優良株の検索

実験方法

a. 菌株の分離法： 研究室周辺の土壌，空気などを分離源とし，平板培養法によって糸状菌だけを選択的に分離採取した．この際，分離用培地としては主として Czapeck-寒天培地を使用した．

b. 優良株の検索法： 各分離菌株をすべて同じ条件の下で培養し，各培養においてどのようなインドール反応陽性物質がどの程度まで生産されているかをしらべた．この場合，斜面はマンニット 3%，ブドウ糖 1%，コハク酸 1%，KH_2PO_4 0.1%，$MgSO_4 \cdot 7\,H_2O$ 0.03% の組成の水道水溶液にアンモニア水を加えて pH 5.6 とするとともに，寒天を 2% の割合に溶解させ，その 5ml

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ずつを径 1.6 cm，長さ 17.5 cm の試験管に分注，殺菌して調製した．また，培養はこれら斜面の全表面に各分離菌株を胞子あるいは菌糸片をもって接種し，28～30℃の恒温室内に約 10 日間放置してこれを行なった．さらに，目的物質については，各斜面培養の試験管内に 1/10 N 硫酸液 10 ml ずつ入れ，培養物全体をこの酸液とかきまぜるとともに 1 夜放置して，培養物中に含まれる塩基性分を酸液中に溶出させたのち，まずその溶出液の一部についてインドール反応の有無を *p*-dimethylaminobenzaldehyde-硫酸液（Allport-Cocking 試薬[5]）によってしらべ，その反応が明瞭に認められた場合には，つぎにその溶出液の残部について，これからそのインドール反応陽性塩基性物質をアンモニア・アルカリ性下で酢酸エチルに転溶，濃縮したのち，その成分を薄層クロマトグラフィーによってしらべた．なお，このクロマトグラフィーは吸着剤としてワコーゲル B-5（厚さ 0.25 mm），展開剤としてクロロホルム-エタノール混液［10：1 (v/v)］を用い，上昇法によって実施した．これによって得られたクロマトグラムは，まずこれを紫外線下で蛍光像の有無をしらべ，ついでこれに *p*-dimethylaminobenzaldehyde 2 g を濃塩酸 20 ml とエタノール 80 ml との混液に溶解して調製した試薬（Ehrlich 試薬）を噴霧して呈色像の有無，その色調などをしらべた．

実験結果

かなり多数の斜面培養においてなんらかのインドール反応陽性塩基性物質が生産されている事実が見出された．なかでも，実験室周辺の空気中から分離採取した菌株中のペニシリウム属の 3 つの菌株の培養においては，麦角アルカロイド様の塩基性物質が著量に生産されている事実が認められ，同時にその塩基性物質がいずれも実施の薄層クロマトグラフィーで，主として *Rf* 0.50，0.41 および 0.02 の各位置に青紫色ないし赤紫色の像として現われる 3 種の物質から成り立っていることが見出された．一方，これら 3 種の物質を量的に多く生産されているものから順に "X" (*Rf* 0.41)，"Y" (*Rf* 0.50) および "Z" (*Rf* 0.02) と仮りに名づけ，それぞれの薄層クロマトグラフィーにおいて示す性質をしらべたところ，"Z" が麦角アルカロイド chanoclavine-(I) と類似した挙動を示すのに対して，"X" と "Y" とは costaclavine[6~8] を含むどの種の既知麦角アルカロイドとも明らかに異なる挙動を示すことが知られた．

II. 検索されたペニシリウム属3菌株の同定

実験方法

常法に従い，各菌株の slide culture（培地：Czapek-寒天）と巨大集落（培地：Czapek-寒天および坂口-上氏-寒天）とについて形態学的諸性質をしらべた．

実験結果

検討したペニシリウム属3菌株のいずれにおいても同様に，1) 菌糸体の大部分が垂直双輪性状である，2) 集落は被子器および菌核を形成せず，また結束糸をも形成しない，3) 37℃ の下では生育が不能である，4) 集落は小さく（26℃，14 日間の培養で直径 3.3～3.5 cm），その表面はビロード状を呈し，裏面は無色，ときには部分的に黄色を呈す，5) 分生胞子は長楕円形で，その表面は平滑である，などの諸性質が見出された．これらを Raper & Thom[9] および Abe[10] の分類に照らしてみたところ，*Penicillium concavo-rugulosum* ABE について記載されているところと全く一致した．さらに，ここに見出された諸性質は，財団法人発酵研究所から譲り受けた *Penicillium concavo-rugulosum* ABE IFO 6226 について観察されるところともよく一致した．

III. *Penicillium concavo-rugulosum* の培養物からインドール反応陽性塩基性物質 "X"，"Y" および "Z" の分離

a. 供試菌株： 前項の実験で検索された *Penicillium concavo-rugulosum* の各株から，コロニー分離法によって多数の菌株を分離し，それらのうちから静置培養で目的の3物質を多量に生産しうる優良株1株を選択してこれを用いた．

b. 培養法： 培養はすべて静置培養法によってこれを行なった．この場合，培地としては種々検討した結果に基づいて，ソルビット 10%，ブドウ糖 4%，コハク酸 1%，KH_2PO_4 0.1%，$MgSO_4 \cdot 7H_2O$ 0.03%，C. S. L. 0.1% の組成の水道水溶液をアンモニア水で pH 5.6 に調整したのち，700 ml のルー・フラスコに 150 ml ずつ分注，殺菌したものを用いた．すなわち，これらの培地に供試菌株の胞子を殺菌水に懸濁して接種し，すべてを 28～30℃ の恒温室内に 15 日間静置した．

c. 抽出法： 上記の培養 80 個をまず菌体と培養液とに濾別し，菌体（乾物量として 120 g）はこれを適量の 1/10N 硫酸液とふりまぜて，その含有ないし付着する塩基性物質をほぼ完全に溶出した．ついでこの溶出液を培養濾液（約 10.8 *l*）と合併し，アンモニア水でアルカリ

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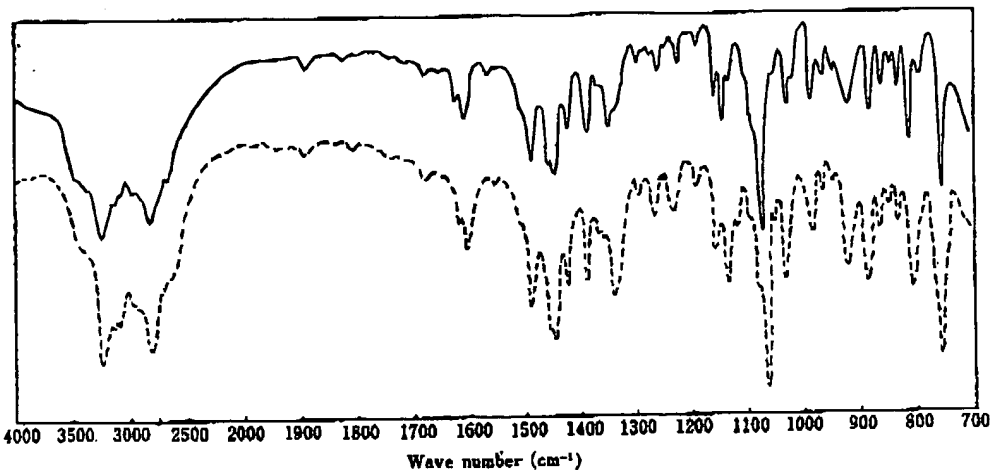

Fig. I. IR-Spectrum of Isolated Substance in KBr.
—— Isolated substance, ---- Chanoclavine-(I)

性としたのち，酢酸エチルとくりかえしふりまぜて，塩基性物質をできるだけ完全に酢酸エチル中に転溶した．こうして得たすべての酢酸エチル溶液を集め，減圧下で濃縮してついに乾固させ，総塩基性物質の粗抽出物約8gを得た（収量，培養瓶1個当たり約100mg）．さらに，この抽出物のすべてを1%リン酸水溶液200mlに溶解し，これを1/10N水酸化ナトリウム溶液でpH 6.0に調節するとともに，クロロホルム毎回200mlとくりかえし4回ふりまぜて，総塩基性物質中の"X"と"Y"とをほぼ完全にクロロホルム中に移行させ，"Z"をリン酸緩衝液中に残留させた．

（i）"Z" [Chanoclavine-(I)] の分離： 上記の"Z"を含むリン酸緩衝溶液をアンモニア水でpH 10前後に調節したのち，酢酸エチル200mlとくりかえし数回ふりまぜて，"Z"をできるだけ完全に酢酸エチル中に転溶した．この酢酸エチル溶液を集め，減圧下でほぼ半量になるまで濃縮したのち，今度はこれを1/10N硫酸液50mlと激しくふりまぜ，"Z"のすべてを酸液中に転溶した．この酸溶液をふたたびアンモニア水でpH 10前後とするとともに，ほぼ等量の酢酸エチルとくりかえしふりまぜて，"Z"をできるだけ完全に酢酸エチル中に転溶した．この酢酸エチル溶液を集め，芒硝で脱水したのち減圧下で5ml程度にまで濃縮し，低温（5°C）の下に放置したところ，目的の物質"Z"が無色の柱状結晶として110mgだけ析出した．本物質はベンゼン，酢酸エチル，アセトン，エタノールなどから特徴ある無色の柱状結晶として析出し，mp 220°C (decomp.)，$[\alpha]_D^{22}$ −234°，$[\alpha]_{546}^{22}$ −286° (c=0.5, pyridine) を示した．また元素分析の結果はつぎのとおりで，分子式は$C_{16}H_{20}ON_2$に相当した．Found：C, 74.99；H, 7.69；N, 10.80. Calcd. for $C_{16}H_{20}ON_2$：C, 74.96；H, 7.86；N, 10.93 %.

本物質は各種の酸類に容易に溶解し，その溶液はAllport-Cocking試薬によって青紫色の反応を与えた．ベンゼン，エーテル，クロロホルムなどにはやや難溶であったが，ピリジンにはもちろん，酢酸エチル，アセトン，メタノールなどにもよく溶解し，これらのどの溶媒もほとんど蛍光を放たなかった．本物質はこれらの諸性質およびその赤外線吸収スペクトル（Fig. 1参照）から容易に麦角アルカロイドChanoclavine-(I)と同定された．

（ii）"X"(Rugulovasine A) の分離： 前記の"X"と"Y"とを含有するクロロホルム溶液全部を集め，これを減圧下で100ml程度にまで濃縮したのち，ほぼ等量の1%リン酸水溶液と激しくふりまぜて，"X"とともに"Y"をも酸液中に転溶し，ついでこの酸溶液をアンモニア水でpH 10前後に調節するとともに，ほぼ等量の酢酸エチルとくりかえし数回ふりまぜて，塩基性物質をできるだけ完全に酢酸エチル中に移行させた．この酢酸エチル溶液を集め，減圧下で乾固するまで濃縮し，その残留物をクロロホルム100mlに溶解した．このクロロホルム溶液についてふたたび塩基性物質のリン酸水溶液への転溶，さらに酢酸エチルへの転溶を行なったのち，この酢酸エチル溶液を集め，芒硝で脱水した．これを減圧下で濃縮して乾固させ，その残留物を少量の熱ベンゼンに溶解したのち室温に放置したところ，目的物質

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"X" の粗結晶 1.05g が析出した. これを濾取し, ベンゼンからくりかえし再結晶して, "X" を分解点 138℃ を示す無色の針状結晶として得た.

本物質は, その別項に述べるような理化学的, 生理的諸性質から, つぎの "Y" との間に相互に交換しうる新規アルカロイドであることが知られ, これを Rugulovasine A と名づけた.

(iii) "Y" (Rugulovasine B) の分離 : 上記のベンゼン溶液から "X" を結晶として析出させた母液中には, 目的物質 "Y" が少量の "X" とともに多量に含有されていたので, これから減圧下で溶媒を留去したのち, その残留物をクロロホルムとリン酸緩衝液とを用いる向流抽出に付し, "Y" だけが含有される部分をうるとともに, それについて種々の方法で "Y" の結晶化を図ってみたが, どの方法によっても "Y" が樹脂状に得られたに過ぎず, ついにその目的を達することができなかった.

一方, 上記の母液からベンゼンを留去した残留物を温アセトン 5ml に溶解し, これに濃塩酸 0.5ml を加え, よくふりまぜたのち室温に放置したところ, "Y" の塩酸塩 1.0g が結晶として析出した. これを採取し, 熱水から再結晶して "Y" の塩を分解点 217℃ の白色柱状結晶として得た. このように, "Y" についてはこれを結晶としてうることができなかったが, その樹脂状精製物および上記塩酸塩などの示す別項のような諸性質から, "Y" そのものは "X" (rugulovasine A) の 1 つの互変異性体であることが確認されたので, これに rugulovasine B の名を与えた.

IV. Rugulovasine A ("X") および B ("Y") の相互変換

前項の実験過程で, "X" および "Y" が二, 三の有機溶媒中で, それぞれ "Y" および "X" に変化する事実がクロマトグラフィーによって認められ, "Y" についてはその有機溶媒溶液が "X" の結晶を与える事実すら認められたが, このことから, この相互変換に関して 1 つの実験を行なってみた.

実験方法

Rugulovasine A の精結晶と rugulovasine B の樹脂状精製物とについて, これらを個々に適当量ずつ種々の有機溶媒に溶解するとともに, 各溶媒の沸点下で 5 分間ずつ処理し, その処理液を吸着剤としてワコーゲル B-5 を用い, 展開剤としてクロロホルムとエタノールとの混液 [10 : 1 (v/v)] を用いる前記の薄層クロマトグラフィーに付し, Ehrlich 試薬を噴霧したのち, rugulovasine A および B に相当する呈色部分をデンシトメーター (OZUMOR-82 型) によって比色定量した.

実験結果

溶媒の種類によって変換状態に著しい差異のあることが認められたが, その代表的なものをとりまとめて示すと Table I のごとくである. すなわち, これにより rugulovasine A と B とは, 条件によってはきわめて容易に相互に変換しうる化学構造を有しているものであることが知られた.

Table I. Conversions of Rugulovasine A and B in Organic Solvents

Solvent	Conversion (%)	
	from Rugulovasine A to B	from Rugulovasine B to A
Ethanol	47	12
Ethyl acetate	28	1
Benzene	13	6
Pyridine	0	0

Each sample was dissolved properly in the solvents, and the solutions were boiled for 5min. The resulting solutions were subjected to thin-layer chromatography on WAKOGEL B-5, using chloroform-ethanol mixture [10 : 1(v/v)]as developing solvent. The chromatograms were investigated with an automatic spectrophotometer, OZUMOR-82, to determine the amounts of alkaloids. The alkaloidal spots were detected with Ehrlich's reagent.

V. 各種糸状菌株の rugulovasines 生産性

実験方法

どの種の糸状菌株をもすべて同じ条件の下で培養し, それらから総塩基性物質分を抽出するとともに, 各抽出物中に rugulovasine A および B が含有されているかどうかを薄層クロマトグラフィーによってしらべた.

a. 被検菌株 : *Aspergillus fumigatus*[11,12] 株のほか, さきに麦角アルカロイド生産性があるものとして検索された 19 種の既知糸状菌株[1]. なお対照として, これらの菌株に前記 *Penicillium concavo-rugulosum* 株中の rugulovasine 生産性の比較的優れた 1 株を加えた.

b. 培養法 : 培地としてはマンニット 3%, ブドウ糖 1%, コハク酸 1%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.03% の組成の水道水溶液をアンモニア水で pH 5.6 に調整したのち, 700ml のルー・フラスコに 150ml ずつ分注, 殺菌したものを用いた. また, 培養はこれらの培地に被検菌株 (馬鈴薯寒天斜面上, 30℃ の下で 10 日間

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生育させたもの)*の胞子あるいは菌糸片を接種し，30°Cの恒温室内に約30日間静置してこれを行なった．

c. **総塩基性物質の抽出法**： 各培養物から，前項Ⅲ．の場合と同様の方法によってインドール反応陽性塩基性分をできるだけ完全に抽出した．この抽出物中には若干の色素や脂肪類が含まれていたが，これをそのまま濃縮して薄層クロマトグラフィーの試料とした．

d. **薄層クロマトグラフィー**： 前項の諸実験の場合と同じ方法によってこれを行なった．ただし，呈色像の *Rf* 値，色調その他から，試料中に多少とも rugulovasines が存在すると認められた場合には，その試料をベンゼン-メタノール混液 [2：1 (v/v)]，クロロホルム-ジエチルアミン混液 [10：1 (v/v)] などを展開剤として用いるクロマトグラフィーにも付し，rugulovasines 標品との異同を吟味した．

実験結果

検討した20種の既知糸状菌株中，*Penicillium concavo-rugulosum* ABE のほか，4種の菌株，*Penicillium rugulosum* THOM，*Corticium caeruleum* (Schrad.) Fr.，*Pellicularia filamentosa* (Pat.) ROGERS および *Lenzites trabea* Fr. の各培養において rugulovasine A および B の少なくとも一方が，chanoclavine-(I) およびその他の麦角アルカロイドまたは麦角アルカロイド様物質とともに生産されている事実が見出され，これらの菌株に rugulovasines 生産性のあることが確認された．

VI. Rugulovasine A および B の理化学的性質

Rugulovasine A： 本物質はベンゼン，クロロホルム，アセトニトリルなどから無色の針状または柱状結晶として析出し，mp 138°C (decomp.)．$[\alpha]_D^{22}$ 0°，$[\alpha]_{546}^{22}$ 0° (c=0.5, pyridine) を示す．また紫外線吸収スペクトルは UV λ_{max}^{EtOH} mμ (ε)：224 (27,040)，277 (5757)，288 (6743)，295 (6682) である．

元素分析，分子量測定の結果はつぎのとおりで，本物質は分子式 $C_{16}H_{16}O_2N_2$ に相当する．

Found：C, 70.83；H, 6.09；N, 10.01；(C) CH_3, 5.54；(N) CH_3, 5.41；mol. wt., 268 (MS)．Calcd. for $C_{16}H_{16}O_2N_2$：C, 71.62；H, 6.01；N, 10.44；(C) CH_3, 5.60；(N) CH_3, 5.60%；mol. wt., 268.3.

各種の酸類に容易に溶解し，その溶液は Allport-Cocking 試薬によって時間の経過とともに赤紫色から青紫色に変化する呈色反応を与える．また石油エーテル，シクロヘキサンなどに難溶；エーテル，クロロホルム，アセトニトリル，ベンゼンなどに可溶：エタノール，酢酸エチル，アセトン，ピリジンなどには易溶である．これらのどの有機溶媒溶液もほとんど螢光を放たない．本物質はこれらの有機溶媒中，とくにエタノール中で容易に rugulovasine B に変化する．水には僅かにしか溶解しないが，その溶液は微アルカリ性の反応を与える．なお，この溶液はイソニトリル反応を示さないが，Dragendorff 試薬によっては橙色の反応を与える．

一方，本物質は種々の無機あるいは有機酸類と容易に結晶性の塩類を形成する．その塩酸塩は水から mp 225°C (decomp.)，$[\alpha]_D^{22}$ 0° (c=1.0, pyridine)，組成 $C_{16}H_{16}O_2N_2 \cdot HCl$ を示す無色の柱状結晶として，またそ

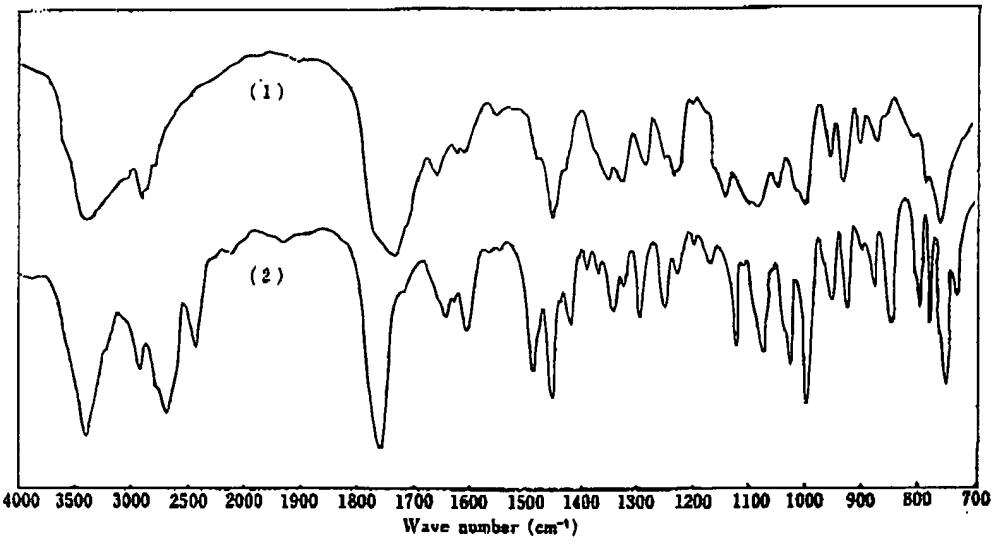

Fig. 2. IR-Spectra of Rugulovasine A (1) and Its Hydrochloride (2) in KBr.

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のピクリン酸塩はメタノールから mp 229℃ (decomp.), $[\alpha]_D^{22}$ 0° (c=1.0, pyridine), 組成 $C_{16}H_{16}O_2N_2 \cdot C_6H_3O_7N_3$ を示す黄色の塊状結晶として, さらにその修酸塩は水から mp 224℃(decomp.), $[\alpha]_D^{22}$ 0° (c=1.0, pyridine), 組成 $C_{16}H_{16}O_2N_2 \cdot C_2H_2O_4$ を示す無色の針状結晶として, それぞれ析出する. rugulovasine A の赤外線吸収スペクトルを, その塩酸塩のそれとともに示すと Fig. 2 のごとくである.

Rugulovasine B: 本物質は水, 石油エーテル, シクロヘキサンなどの難溶性溶媒以外の各種溶媒にきわめて易溶なため, 遊離の塩基としては結晶されにくい. これらの易溶性溶媒中で rugulovasine A に変化し, 往々その結晶を与える. その樹脂状精製物の紫外線吸収スペクトルは UV λ_{max}^{EtOH} mμ(ε) : 227 (23,600), 278 (5025), 288 (5887), 295 (5833) である. 本物質も酸類とは容易に結晶性の塩類を形成し, その塩酸塩は水から特徴ある白色の柱状結晶として析出し, mp 187℃ (decomp.), $[\alpha]_D^{22}$ 0°, $[\alpha]_{546}^{22}$ 0° (c=0.5, pyridine) を示す. この塩酸塩はつぎのような元素分析値を与え, 組成 $C_{16}H_{16}O_2N_2 \cdot HCl \cdot H_2O$ に相当する.

Found : C, 59.12 ; H, 6.01 ; N, 8.63 ; Cl, 11.43. Calcd. for $C_{16}H_{16}O_2N_2 \cdot HCl \cdot H_2O$: C, 59.53 ; H, 5.89 ; N, 8.68 ; Cl, 11.01%.

質量分析の結果は, 本塩酸塩の塩基 (rugulovasine B) が分子量 268 のものであることを示し, 上記の分子式 $C_{16}H_{16}O_2N_2$ から得られる計算値 268.3 とよく一致する.

また, rugulovasine B の修酸塩は水から mp 217℃ (decomp.) を示す無色の柱状結晶として析出し, ピリジン中で旋光性を全く示さない. 元素分析の結果はつぎのとおりで, この修酸塩は組成 $C_{16}H_{16}O_2N_2 \cdot 1/2\,C_2H_2O_4$ に一致する.

Found : C, 64.40 ; H, 5.51 ; N, 8.64. Calcd. for $C_{16}H_{16}O_2N_2 \cdot 1/2\,C_2H_2O_4$: C, 65.16 ; H, 5.47 ; N, 8.94 %.

これらの塩類は, 遊離の rugulovasine B そのものとはもちろん, rugulovasine A およびその塩類とも同様, どの種の溶媒中でもほとんど螢光を放たず, Allport-Cocking 試薬によって赤紫色から青紫色へと変化する反応を与える. Fig. 3 は rugulovasine B 塩酸塩の赤外線吸収スペクトルを, 遊離の rugulovasine B (樹脂状精製物) のそれと比較して示したものである.

Rugulovasine A, B 両異性体が, どのような構造をもつアルカロイドであるかは明らかでないが, 上記のような諸性質, とくに両物質の赤外線吸収スペクトルが, いずれも不飽和ラクトン (1740～1760 cm^{-1}) および第二級アミン塩酸塩 (2420～2440 cm^{-1}) の吸収を示す事実からみると, 両者は chanoclavines と同様, エルゴリン核のD環が開裂した三環性麦角アルカロイドで, 分子中に不飽和ラクトン環1個を有しているものと考えられる.

VII. Rugulovasine A および B の生理作用

Rugulovasine A, B 両物質が, 麦角アルカロイドに関連した新物質であることから, これらの塩酸塩につい

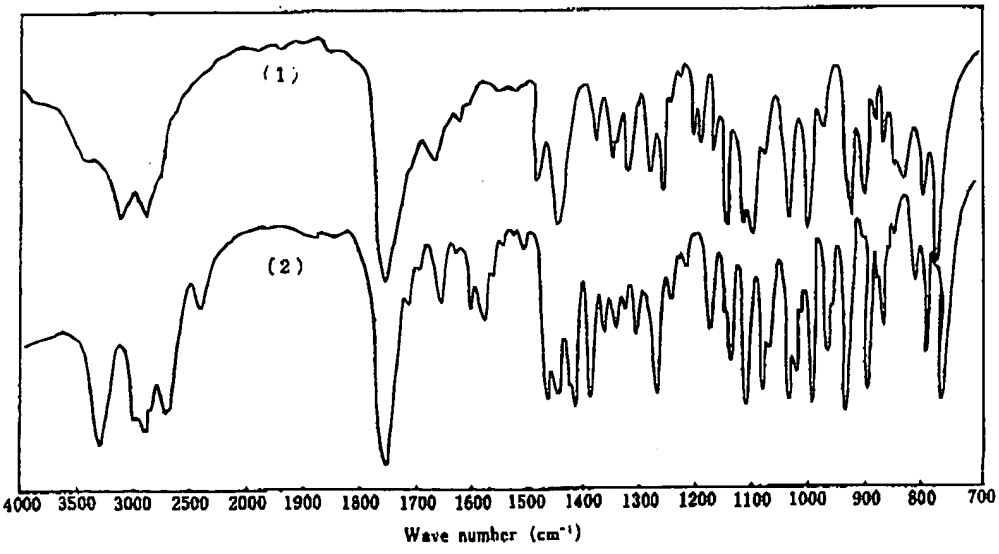

Fig. 3. IR-Spectra of Rugulovasine B (1) and Its Hydrochlorid (2) in KBr.

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て薬理作用をしらべてみた．まず，急性毒性については，供試のどの塩酸塩もマウスにおける静脈注射で，LD_{50} 70～80 mg/kg を示すことが知られた．ついで，循環系その他に対する作用を検討したところ，両物質がいずれも種々の実験動物において中等度の血圧降下作用を示すことが見出された．その最少有効量は，たとえばクロラローゼとウレタンとで麻酔した猫に静脈注射した場合には，rugulovasine A-塩酸塩で 0.2～0.5 mg/kg，rugulovasine B-塩酸塩で 0.025～0.05 mg/kg であった．この場合，rugulovasine A-塩酸塩では，降圧作用が静脈注射後 30 分で現われ，その後 6～7 時間，作用がそのまま持続したが，rugulovasine B-塩酸塩では降圧作用が注射後速やかに現われ，その後約 1 時間で完全に消滅した．

要　　約

実験室周辺の空気中から，静置ならびに振盪培養で麦角アルカロイド様のインドール反応陽性塩基性物質を著量に生産しうるペニシリウム属の3菌株を分離した．これらの菌株がいずれも *Penicillium concavo-rugulosum* ABE であることを明らかにし，同時にその静置培養物から麦角アルカロイド chanoclavine-(I) および2種の新規インドール・アルカロイドを単離した．両新アルカロイドは，ともに分子式 $C_{16}H_{16}O_2N_2$ に相当し，種々の有機溶媒中で相互に異性化し，実験動物に対して血圧降下作用を示したので，これらを rugulovasine A および B と名づけた．rugulovasine A, B 両異性体は，ともにエルゴリン核のD環が開裂した三環性麦角アルカロイドで，分子中に不飽和ラクトン環1個を有しているものと推定された．なお，両異性体は *Penicillium concavo-rugulosum* 株にかぎらず，*Penicillium rugulosum*, *Corticium caeruleum*, *Pellicularia filamentosa*, *Lenzites trabea* など種々の糸状菌株によっても，常に Chanoclavine-(I) およびその他の麦角アルカロイド様物質とともに生産されることが見出された．

終りに，終始御指導，御鞭撻を賜った坂口謹一郎先生に深謝いたします．また，本研究に終始御協力下さった武田研究所大和谷三郎博士および薬理実験をお引き受け下さった同研究所荒蒋義和博士に厚く感謝いたします．

（本研究は昭和43年11月10日，日本農芸化学会関東・支部大会で講演した．）

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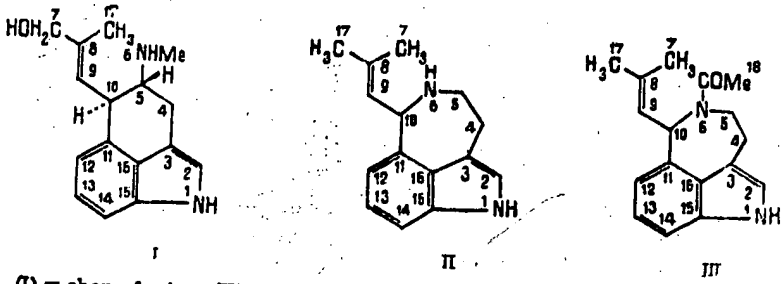
CONFORMATIONS OF THE ERGOT ALKALOIDS CHANOCLAVINE-1, AURANTIOCLAVINE, AND N-ACETYLAURANTIOCLAVINE

V. G. Sakharovskii, A. V. Aripovskii,
M. B. Baru, and A. G. Kozlovskii

UDC 547.94:541.63:543.422.23

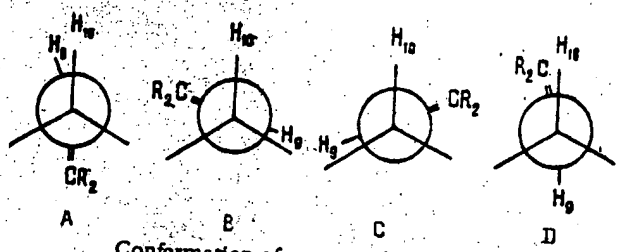
In order to study the conformational states of the ergot alkaloids containing an exocyclic 2',2'-dialkylvinyl grouping, we have performed a comparative study of the ^1H NMR spectra of chanoclavine-1 (I) [1], aurantioclavine (II) [2] and N-acetylaurantioclavine (III), obtained by the acetylation of aurantioclavine with acetic anhydride at room temperature.



I II III

(I) — chanoclavine; (II) — aurantioclavine; (III) — N-acetylaurantioclavine.

The values of $^3J_{9,10}$ calculated from the angular dependence of $^3J_{H,H}$ in the allyl fragment [3] for conformations A, B, C, and D are 6.6, 4.9, 4.9, and 11.5 Hz, respectively. Compounds (I) and (II) possess high values of $\langle ^3J_{9,10}\rangle$ (9.8 and 9.2 Hz, respectively), and can therefore be represented in the form of an equilibrium mixtures of conformers A-D with a predominance of conformer D. The complete absence of homoallyl interaction of H_{10} with the protons of the methyl groups in (I) and (II) also shows the predominance of conformer D [4]. From the condition $\langle ^3J\rangle = \sum_{\varphi} {}^3J_{\varphi} \cdot N_{\varphi}$, where $^3J_{\varphi}$ is the spin-spin coupling constant corresponding to this conformer and N_{φ} is its molar fraction, it is easy to estimate the amount of D in the mixture of conformers (D > 70%). When the orientation influence of the nitrogen atom on $^3J_{9,10}$ in (II) is taken into account [3], the calculated proportion of D becomes even higher.



A B C D

Conformation of compounds (I) - (III) ($R = CH_3$) at the $C_9 - C_{10}$ bond.

The passage from (II) to (III) is accompanied by a marked decrease in $^4J_{10,12}$: thus, for (II), $^4J_{10,12} = 1.3$ Hz, and for (III) $^4J_{10,12} = 0.2$ Hz. This fact can be explained by a change in the conformation of the seven-membered heterocycle with the passage of the $Me_2C{=}CH$ substituent from the pseudoequatorial to the pseudoaxial conformation [4]. This conclusion is also confirmed by the screening of the H_9 proton observed in the passage from (II) to (III) ($\Delta\delta = +0.30$ ppm) with a simultaneous decreasing of H_{10} ($\Delta\delta = -0.98$ ppm).

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An analysis of the $\langle {}^3J_{9,10} \rangle$ values for (III) — 8.1 and 7.5 Hz, respectively, for the Z and E form of the acetamide grouping (the E form was identified from its NOE value of H_{17}—H_{18} = 4%) shows that, in the first place, in contrast to (I) and (II), in (III) the D conformer is no longer predominant and, in the second place, the E form contains an appreciably lower proportion of the D conformer than Z form. The influence of the state of acetamide fragment on the proportion of the D conformer in (III) can be explained in terms of the Van der Waals interaction of the 7-Me group with the 18-Me group (E) and the oxygen atom (Z) of the acetyl residue. An analysis of the calculated values of the energies of these interactions ("6-exp" potential) with Dashevskii's parameters [5]) shows that the D conformation of the side chain of (III) is destabilized by the E conformation of the acetamide grouping by ≈ 0.5 kcal/mole more strongly than the Z conformation.

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ALKALOIDS OF *Delphinium ternatum*

V. M. Matveev, A. S. Narzullaev, and S. S. Sabirov

UDC 547.944/945

Continuing a study of the alkaloid composition of *Delphinium ternatum* [1], we have investigated the epigeal part and roots of the plant collected in the flowering phase on June 18, 1979 in the basin of R. Varzob. The customary chloroform extraction of the epigeal part yielded 0.15% of combined bases, which was separated into phenolic and nonphenolic fractions. Crystals isolated from the ether-soluble nonphenolic fraction proved, on TLC, to be a mixture of two substances. Chromatography on a column of alumina [eluent: hexane—ethyl acetate (1:1)] yielded delcorine [2] and a base (I) with mp 215-217°C (acetone). From the mother liquor after the separation of the crystals, an additional amount of base (I) was isolated by chromatography on a column of alumina [eluent: ether—chloroform (1:25)].

The IR spectrum of (I) is characteristic for diterpene alkaloids of the lycoctonine type, having absorption bands at 1100 cm^{-1} (ether C—O bonds) and 3475 cm^{-1} (hydroxy groups). The NMR spectra ($CDCl_3$, ppm, δ scale, HMDS = 0) revealed signals in the form of a three-proton singlet at 0.86 from a $-C-CH_3$ group; a three-proton triplet at 0.98 from a $>N-CH_2-CH_3$ group; three-proton singlets at 3.16, 3.25, and 3.33 from three methoxy groups; a one-proton triplet at 3.59 from β-H at C-14; a one-proton singlet at 4.11 from α-H at C-6; and two one-proton singlets at 4.92 and 5.02 from a CH_2O_2 group.

Analysis of the results that we had obtained and their comparison with information in the literature enabled base (I) to be identified as delpheline [3-5]. This is the first time that delpheline has been isolated from plants of the family *Ranunculaceae* growing in the territory of the USSR.

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